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Role of B cells in the generation of thymus-derived regulatory T cells

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Supervisor's statement:

I declare, that this PhD dissertation has been prepared under my supervision. I confirm that the dissertation meets the requirements for the official procedure associated with the awarding of a PhD degree in life science, in the area of (biology).

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Co-stimulatory molecules (CD80, CD86, CD40)

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Dedication

This dissertation is dedicated to my wonderful and loving family; firstly my wonderful parents (father and mother), who without their prayers and encouragement I could not get to this happy end. And to the most wonderful wife, Wasan, who has always encouraged and supported me throughout my study, and to the most beautiful children, Nabaa and Karrar, who bring me the greatest joy and are my constant source of determination and give me the will to succeed. To my brother dr. Adnan Hashim Muhammed Ali for their love and constant encouragement and support over the years. To my sisters Hanna and Asmaa who inspire me each day of my life.

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List of abbreviations

Ag (antigen)

Aire (autoimmune regulator)

APC (antigen-presenting cell)

BCR (B cell receptor)

cDC (thymic classical dendritic cell)

CFSE (carboxyfluorescein diacetate succinimidyl ester)

cTEC (cortical thymic epithelial cell)

CTLA-4 (cytotoxic T-lymphocyte-associated protein 4)

DC (dendritic cell)

Dex (dexamethasone)

DN (double-negative subset)

DNA (deoxyribose nucleic acid)

DP (double-positive subset)

DST (donor-specific transfusion)

EAE (Experimental autoimmune encephalomyelitis)

Ebi3 (Epstein-Barr virus-induced gene 3)

Foxp3 (forkhead boxp3 or winged helix transcription factor)

G1 (gap 1)

GC (germinal center)

GFP (green fluorescent protein)

GITR (glucocorticoid-induced TNFR-related protein)

GvHD (Graft-versus-Host Disease)

HSC (haematopoietic stem cell)

ICAM-1 (Intercellular Adhesion Molecule 1)

IDO (indoleamine 2,3-dioxygenase)

IEL (intestinal intraepithelial lymphocytes)

IFN-γ (Interferon gamma)

Ig (immunoglobulin)

IL (interleukin)

IMQ (Imiquimod)

IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome)

iTreg (induced or adaptive regulatory T cell)

IVC (individually ventilated cages)

LAG-3 (Lymphocyte-activation gene 3)

LD (light/dark)

LFA-1 (Lymphocyte function-associated antigen 1)

LPS (lipopolysaccharide)

MACS (Magnetic-activated cell sorting)

MFI (Mean Fluorescent Intensity)

MHC (major histocompatibility complex)

Mls (minor lymphocyte-stimulating)

mRNA (messenger RNA)

mTEC (medullary thymic epithelial cell)

NK (natural killer cell)

NKT (natural killer T cell)

NOD (non-obese diabetes)

NRP-1 (Neuropilin-1)

nTreg (naturally occurring regulatory T cell)

PAMP (pathogens associated molecular pattern)

PBS (phosphate-buffered saline)

PD1 (programmed cell death 1)

PD-L1 (Programmed death-ligand 1)

PRR (patterns recognition receptor)

RA (Retinoic Acid)

RBC (red blood corpuscle)

RNA (ribose nucleic acid)

SD (Standard Deviation)

SP (single-positive stage)

STAT5 (signal transducer and activator of transcription 5)

Tconv (conventional T cell)

TCR (T cell receptor)

Teff (T effector cell)

TGF-β (transforming growth factor-β)

Th (T helper cell)

TLR (toll-like receptor)

TNFα (tumor necrosis factor alpha)

Treg (regulatory T cell)

Abstract

The importance of natural regulatory T cells (nTregs) characterized by CD4+CD25+Foxp3+ phenotype lies in their ability to suppress pathological and physiological immune response, and control the responsiveness to self-antigens. Thus, nTregs have a pivotal role in the maintenance of self-tolerance and immune homeostasis. They are developed in the thymus and migrate to peripheral lymphoid organs to exert their suppressive function. Their development and activation is dependent on the interaction with antigen-presenting cells (APCs), mainly dendritic cells. B cells can also play a role of APC because of their high constitutive expression of MHC II and the ability to uptake, process and present antigens to CD4+ T cells. The aim of the studies presented in this dissertation was to examine the potential of mouse B cells as APC in the generation of thymus-derived nTregs in an *in vitro* model of co-culture of thymocytes containing nTreg precursors with splenic B cells. Mirroring the role of dendritic cell maturation stage in the differentiation of induced CD4+CD25+Foxp3+ regulatory T cells in lymphoid organs and nTregs in the thymus, we decided to investigate the impact of MHC II and co-stimulatory molecules (CD80, CD86, CD40) expression on B cells upon activation. Imiquimod (IMQ) and lipopolysaccharide (LPS) were used to activate B cells through TLR7 and TLR4, respectively. In addition to (thymic DC and epithelial cell) a small population of B cells can be found in the thymus. Here the role of thymic B cells in the development of nTregs is discussed based on the results of preliminary studies. It is generally accepted that glucocorticoids acting, mainly as immunosuppressive agents, influence dendritic cells maturation by inhibiting the expression of MHC II and co-stimulatory molecules and maintaining the tolerogenic state of antigen-presenting cells. The impact of a synthetic glucocorticoid, dexamethasone (Dex) to induce tolerogenic B cells and to influence the potential of B cells to induce nTreg generation was investigated.

The results of the studies presented in this dissertation are divided into three sections; the first section is related to the role of splenic B cells in thymus-derived regulatory T cells development, the second section is focused on the role of thymic B cells in the development of nTregs, and the third section describes the effect of dexamethasone on the generation of nTregs in the co-culture of thymocytes and splenic B cells. The

results of the study presented in the first section demonstrated that LPS- and IMQ-activated splenic B cells upregulate the expression of MHC II and co-stimulatory molecules that are essential for the presentation of antigens. Both TLR4 and TLR7 activation increased the percentage of B cells positive for particular molecules or their expression facilitating B cells to interact with CD4+ T cells.

Co-culture of thymocytes with splenic B cells changed the pattern of the distribution of the main thymocyte subsets mainly DN CD4–CD8– and DP CD4+CD8+ with minor differences in the percentage of SP CD4+CD8– and CD8+CD4– independently on thymocytes : B cells ratio and B cell activation. However, in the microenvironment influenced by the presence of B cells the percentage of SP CD4+Foxp3+ thymocytes was increased. This phenomenon was dependent on the activation of B cells and thymocytes : B cells ratio. Thus, splenic B cells demonstrated the potential to generate nTregs *in vitro* dependently on the strength of co-stimulatory signals provided to developing thymocytes.

In addition, the presence of splenic B cells resulted in the increase of Foxp3 transcription factor expression. The biological activity of nTregs generated in this study was investigated. It was demonstrated that nTregs isolated from cultures maintained under different conditions statistically decreased the percentage of proliferating, activated, responder CD4+ T cells. Blockade of co-stimulatory molecules and MHC II on B cells revealed that the implication of CD80/CD86 and MHC II molecules was more efficient than CD40 molecule in the process of nTreg generation by B cells indicating to the importance of engagement of CD80/CD86 with CD28 molecule, while the engagement of CD40/CD40L is less important in this process.

The role of thymic B cells in the generation of nTregs *in vitro* was presented in the second section. The results of 72 hours of culture revealed that activated thymic B cells maintained the level of nTreg generation independently of the activator used (LPS or IMQ), or counteracted the decrease of these cells observed in non-activated culture. The results revealed that thymic B cells differ from splenic B cells by their characteristics upon activation; in addition, activation by high concentration of LPS (LPS high), or low concentration of imiquimod (IMQ low) induced the increase of thymic B cells about 2-3-fold after 72 hours of culture compared to 24 hours of culture. This observation allows us to conclude that thymic B cells and splenic B cells

influence the generation of nTreg in different ways. In addition, the activated cultures showed increased expression of Foxp3 transcription factor.

The aim of the studies presented in the third section was to investigate the potential of Dex to render B cells tolerogenic and in consequence to facilitate the generation of nTregs. The results presented in this section showed that Dex induced the decrease of percentage of positive B cells for co-stimulatory molecules as well as for MHC II. Dex did not change the distribution of thymocyte subsets nor did it influence the generation of thymus-derived nTreg cells.

1. Introduction

The function of the immune system is to protect an individual from invader pathogens such as bacteria, viruses, fungi, parasites or other potential threats, while maintaining unresponsiveness against self-antigens or non-infectious agents (Parkin and Cohen, 2001; McHugh and Shevach, 2002; Powrie et al., 2003). However, our environment contains many other harmless organisms which can activate the immune system in a positive protective manner. In addition, the immune system must be able to discriminate between "self" and "non-self" to avoid attacking self-systems that might lead to autoreactivity resulting in autoimmune diseases. Therefore, a balance between tolerance to harmless and self-antigens and immunity against pathogens must be maintained (Banchereau et al., 2000; Shklovskaya and Fazekas de St Groth, 2007; Cools et al., 2007; Novak and Bieber, 2008).

In vertebrates, the immune system is composed of innate (nonspecific) and adaptive (specific) immunity, arising from bone marrow-resident haematopoietic stem cells (HSC) (Ghia et al., 1998; Monson, 2008). The innate immunity is composed of mucous membranes, phagocytes and cytotoxic cells which provide non-specific first line defense mechanisms against pathogens. Its function is based on the recognition and binding of pathogens associated molecular patterns (PAMPs) through germline encoded patterns recognition receptors (PRRs), of which the best known are toll-like receptors (TLRs), expressed mainly by different types of antigen-presenting cells (APCs) such as macrophages and dendritic cells (Iwasaki and Medzhitov, 2004). PAMPs are specific conserved structures, such as lipopolysaccharide (LPS) from Gram-negative bacteria, which are components of bacterial cell wall. Signaling through TLR induces cascade of signals, which ultimately leads to rapid clearance of the infection (Akira and Hemmi, 2003). Pathogens that overcome this initial immune response are confronted by more specific line of defense, namely adaptive immunity (Paul, 2003).

Adaptive immunity is composed of CD8+ T cells, CD4+ T cells, NKT cells, and B cells expressing clonally rearranged receptors activated after antigen encounter (Abbas et al., 2005). After pathogen clearance, most of the antigen-specific T and B cells undergo apoptosis while a few of them enter a memory pool; this ensures a quicker and stronger reaction during secondary infection, a process called memory (Yoshida et al., 2010; Li et al., 2011).

Rearrangement of T and B cell receptors is completely random and rearrangement products can be potentially self-reactive, therefore it is a vital requirement for the immune system to eliminate or control auto-reactive T and B cell clones in a process called tolerance. There are two different stages of tolerance : *central tolerance* that defines mechanisms of clonal deletion or receptor editing of developing T or B cell (Kappler et al., 1987; Kisielow et al., 1988; Nemazee and Burki, 1989; Boehmer, 2009), and *peripheral tolerance* that refers to the control of survival and activity of auto-reactive lymphocyte that escaped central tolerance (Ashour and Niederkorn, 2006).

1.1. Innate and adaptive immunity

Innate immunity is triggered upon pathogen recognition by PRRs. The most explored group of PRRs is the family of TLRs recognizing conserved molecular patterns shared by groups of microorganisms or viral and bacterial RNA and DNA. In humans, there are 11 known TLRs recognizing different PAMPs. For instance, TLR4 binds to bacterial lipopolysaccharide found in Gram-negative bacteria cell wall, while TLR7 and TLR8 bind to single-stranded RNA in viruses (Medzhitov and Janeway, 2000). So far 13 mammalian TLRs have been identified (McGettrick and O'Neill, 2007). Both humans and mice express TLR1–9, TLR10 is specific for humans only, whereas TLR11–13 are specific for mice (Chaturvedi and Pierce, 2009). Corresponding to the class of recognized ligands, TLRs are expressed in two distinct cellular compartments. In humans, TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the outer cell membrane and recognize mainly bacterial surface-associated PAMPs such as peptidoglycan and lipopeptides (TLR1, 2, 6), lipopolysaccharide (TLR4), and flagellin (TLR5). The other human TLR are expressed on the membrane of intracellular endosomes and bind to viral dsRNA (TLR3), ssRNA (TLR7 and 8), and unmethylated bacterial DNA (TLR9) (Chaturvedi and Pierce, 2009; Takeda and Akira, 2005).

One of the major contributions of the innate immunity is the barrier function of the epithelial surfaces preventing entry of microbes into the body. Other mechanisms include mucin production by certain epithelia, and release of anti-microbial factors such as defensins, lysozyme, and lactoferrin (Ouellette, 1989;1994; Mestas and Hughes, 2004). Mucins act as a highly viscous, protective layer entrapping microorganisms on the apical surface of epithelial cells. Antimicrobial peptides can be produced constitutively or induced by microbial molecules binding to TLRs.

Any infectious agent crossing the epithelial barrier should be recognized by TLRs on resident macrophages and induce a cascade reaction leading to elimination of the invading pathogen through various mechanisms including phagocytosis and intracellular killing of the microorganism by macrophages and neutrophils, or direct killing by defensins or lysozyme. Another important innate immunity mechanism is activation of the complement system via alternative or lectin pathways. This leads to the deposition of C3b on the surface of the microorganism, which acts as an opsonizing agent promoting microbial uptake by phagocytic cells. As a consequence of complement activation membrane-attack complex is formed that results in lysis of the pathogen. Phagocytic cells release different cytokines, which in turn can induce the mobilization of antigen-presenting cells (APCs) that are important for the induction of the adaptive immunity (Male et al., 2000).

Lymphocytes are the central players of adaptive immunity. There are two main populations of lymphocytes: T lymphocytes (T cells) responsible for cell-mediated immunity and immune regulation (McHugh and Shevach, 2002; Powrie et al., 2003; Fujio et al., 2010; Luckheeram et al., 2012), and B lymphocytes (B cells), responsible for humoral immunity (Youinou , 2007; Vitale et al., 2010) . In contrast to innate immunity, adaptive immunity is highly specific and is able to recognize small structural fragments (called epitopes) on foreign molecules (antigens). It is also highly effective in complete elimination of the invading micro-organism. Circulating antibodies are the effector molecules of humoral immunity. They bind to epitopes of foreign antigens and contribute to the elimination of pathogens in various immune reactions. In cell-mediated immunity the effectors are cytotoxic CD8+ T cells and a variety of cytokine producing T cell populations.

B and T cells utilize highly specific receptors. B cells use cell-membrane bound immunoglobulin (mIg) molecules (BCRs, B Cell Receptors). Upon activation B cells proliferate, differentiate into plasma cells, and secrete immunoglobulins (soluble antibodies) providing defense against pathogens in the extra-cellular spaces of the body. The largest population of T cells, Tαβ cells, have specific receptors (TCRs, T Cell Receptors) that recognize small peptides derived from protein antigens presented on the surface of antigen-presenting cells (APCs) in context of major histocompatibility complex molecules (MHC). MHC I-peptide complexes are recognized by TCRs of CD8+ T cells, while CD4+ T cell TCRs bind to MHC II-peptides. Depending on the type of activated T cells, the activation might result in

killing of infected target cells by cytotoxic CD8+ T cells, activation of macrophages and B cells by different effector helper CD4+ T cells or inhibition of the activity of various adaptive and innate immune cells by regulatory T cells induced upon activation.

The adaptive immunity is highly specific and each encounter of a new foreign antigen will induce long-lived specific memory cells that will protect an individual from re-infection with the same pathogen. Memory cell repertoires are changing during the life span of individual resulting in increased protection throughout the life and tend to decrease when the immune system starts to senesce. Innate immune responses recognize generic targets on pathogens using germ line encoded receptors, whereas adaptive immune response recognizes specific targets using randomly generated receptors which have a virtually unlimited recognition repertoires.

1.2. Regulatory T cells: phenotype, origin and suppressive mechanisms

It is very well documented that regulatory T cells (Tregs) are involved in the maintenance of tolerance and immune homeostasis. In one of the recent papers, Sakaguchi classifies the mechanisms of tolerance and immune homeostasis as recessive and dominant (Sakaguchi et al., 2008). In the recessive mechanisms, the fate of autoreactive lymphocyte exposed to self-antigen is determined by intrinsic manner. Most immature thymocytes are programmed to die by apoptosis when exposed to self-antigens associated with MHC. The activation threshold of thymocytes and T cells in peripheral lymphoid organs may be increased by the induction of negatively regulating proteins (inhibitory receptors, negative signaling molecules). On the other hand, the cells may not survive because of activation induced cell death. This cell-intrinsic control of the cell fate also contributes to the inhibition of an excessive response to non-self/conventional antigens. In dominant (extrinsic) mechanism requires T cells with specialized suppressive activity, namely T regulatory cells. In addition, it is considered that every immune response generates both effector and suppressive cells.

1.2.1. Phenotype and origin

There are two populations of Tregs distinguished on the basis of generation site (Fig. 1.1): thymus-derived Tregs (tTregs) also named natural Tregs (nTregs) developed in the thymus and peripherally-derived Tregs (pTregs) known also as induced Tregs (iTregs) that differentiate from conventional naïve CD4+Foxp3− T cells after antigen exposure under particular conditions in peripheral lymphoid organs

(Sakaguchi et al., 1995; King and Sarvetnick, 1997; Groux et al., 1997; Weiner, 2001; Elkord, 2014). Natural regulatory T cells are developed in the thymus as functionally mature suppressive T cell population, whereas iTregs are generated in the periphery after cognate antigen challenge (Wing and Sakaguchi, 2010). Many cell surface markers were described as expressed by nTregs but there is no specific molecule considered as specific marker to distinguish them from iTregs in peripheral lymphoid organs.

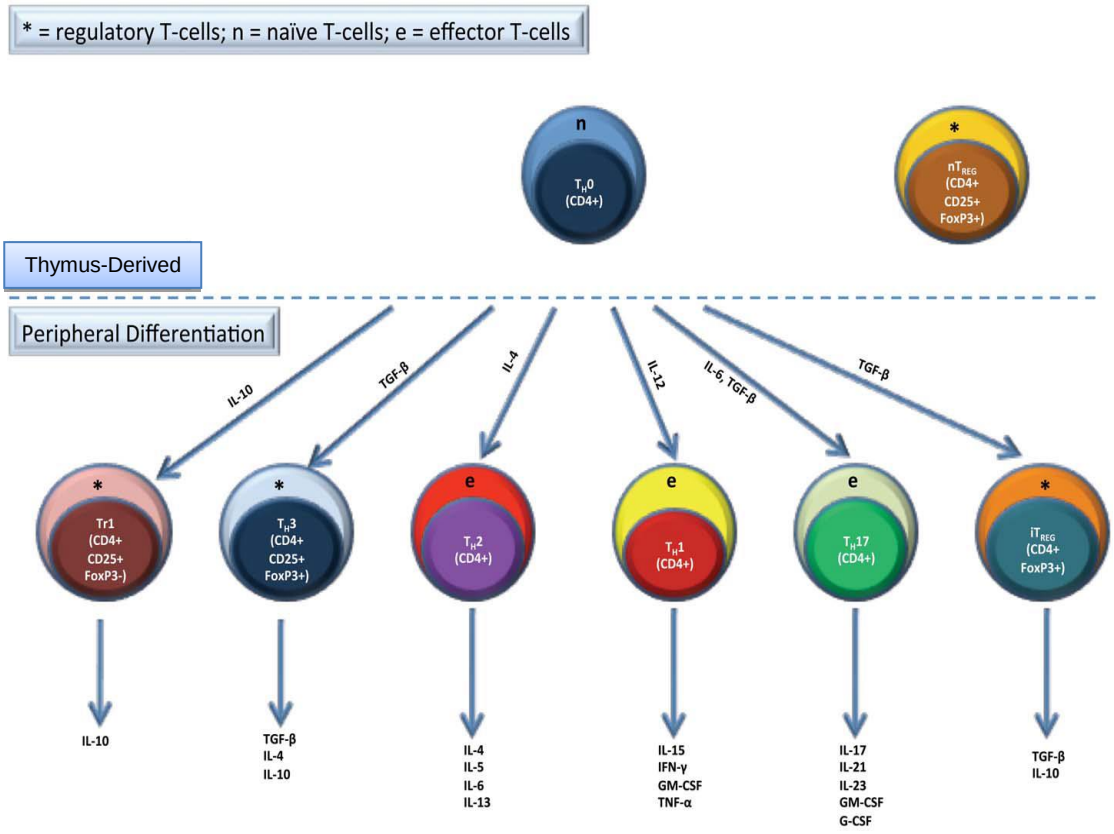


Figure 1.1:—Populations of Tregs and effector T helper cells depending on the site of generation. Tregs are either generated naturally by thymic differentiation (nTreg) or are induced in the periphery (iTreg) from naïve T helper cells (T_H0, naive helper T cells). Subpopulations of iTreg include T_H3 inhibiting the immune response mainly through TGF-β and interacting with B cells to enhance or inhibit their activity, Tr1 suppressing autoimmune response and tissue inflammation partly through secretion of IL-10. The main effectors differentiating from T helper cells include T_H1, T_H2, and T_H17. (Peterson, 2012).

It has been suggested that regulatory T cells are defined by the expression of the forkhead family-transcription factor X-linked forkhead/winged helix (Foxp3). Foxp3 expression is required for regulatory T cell development and function (Fontenot et al., 2003; Khatteri et al., 2003; Hori et al., 2003), and this transcription factor control a genetic program specifying this cell fate (Marson et al., 2007). Foxp3

was initially identified as the gene responsible for an X-linked recessive inflammatory disease in scurfy mutant mice, and further in humans for the fatal autoimmune/inflammatory disease, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Brunkow et al., 2001; Wildin et al., 2001), a severe autoimmune disease that develops early in infancy. In mice, Foxp3 is almost exclusively expressed intracellularly in nTregs and iTregs. In humans, its expression is also detected in peripherally generated regulatory T cells (Fehervari and Sakaguchi, 2004). Although Foxp3 appears to be required for human Treg development and function, expression of Foxp3 alone is not sufficient as a significant percentage of human activated T cells express Foxp3 and yet do not exert suppressive activity (Wang et al., 2007). Furthermore, induction of Foxp3 in human T cells by transforming growth factor-β (TGF-β) does not confer regulatory phenotype, in contrast to their murine counterparts (Tran et al., 2007). The absence of functional Foxp3 results in severe systemic autoimmune diseases in mouse and human. It has been shown that Foxp3 inhibits IL-2 transcription and induces up-regulation of Treg-associated molecules, such as CD25, GITR (glucocorticoid-induced TNFR-related protein) and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) (Shevach and Stephens, 2006), that can down-regulate the immune response of adjacent cells, (Table 1).

Table 1: Molecules expressed by Tregs (nTregs and iTregs) constitutively or upon activation.

Molecule	nTreg	iTreg	Functions	
Foxp3	+	+/ ─	Master regulator (development and function)	Zhang and Zhao, 2007
CD4	+	+	Co-receptor for APCs (interact with MHC II)	Brady et al., 1993
CD25	+	+	High-affinity receptor for IL-2 (IL-2Rα)	Janeway et al., 2001
CD28	+	+	Co-receptor molecule (interacts with CD80 and CD86), transmits an activatory signal to T cell	Harding et al., 1992
CD152 (CTLA-4)	+	+	Co-receptor molecule (interacts with CD80 and CD86), transmits an inhibitory signal to T cell	Walunas et al., 1994

CD278 (ICOS)	?	+/?	Co-stimulatory molecule involved in cell-cell signaling, immune response, and regulation of cell proliferation	Guedan et al., 2014
GITR	+	+	Inhibits suppressive activity of regulatory T-cells and increases survival of effector T-cells	Shimizu et al., 2002
CD69	+	+	Signal-transmitting receptor in lymphocytes involved in lymphocyte proliferation and functions	Tough et al., 1997
CD103 (HML-1, integrin α E)	+	+/?	Important for T cell homing to the intestinal sites	Agace et al., 2000; Huehn et al.,2004; Shimizu et al., 2003
CD38	+	+	Multifunctional ectoenzyme that catalyzes the synthesis and hydrolysis of cyclic ADP-ribose (cADPR) from NAD^+ to ADP-ribose	Read et al., 1998
Fas (CD95)	+	+	Transduces apoptotic signal from T cells to target cells	Suda and Nagata, 1997
CD154 (CD40L)	+	+	Co-stimulatory molecule	Danese et al., 2004
CD223 (LAG-3)	+	?	May have a negative regulatory function.	Workman et al., 2004
CD31 (PECAM-1)	+	─	A member of immunoglobulin superfamily involved in leukocyte transmigration, angiogenesis, and integrin activation	Marson et al., 2007

CD62L (L-selectin, LECAM1)	+	+/?	Adhesion molecule involved in cell activation and circulation, also acts as a "homing receptor" for lymphocytes to enter secondary lymphoid tissues via high endothelial venules	Robbins et al., 1998; Gomes-Pereira et al., 2004
CD127 (IL-7R α-chain)	+	+/?	Involved in T cell proliferation	Seddon and Zamoyska, 2002
CD39 and CD73	+	+/?	CD39 and CD73 ectonucleotidase activity generates pericellular adenosine, which inhibits various activated immune cell types in an A2AR-dependent cAMP- fashion	Bynoe and Viret, 2008; Antonioli et al., 2013
CD45RA, CD45RO	?	+/?	Signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation	Seddiki et al., 2006; Fukuhara et al., 2002
CD49d (VLA4α, Integrin α4)	─	+/ ─	Key mediator of T cell entry into the CNS	Vajkoczy et al., 2001; Kleinewietfeld et al., 2009
CD101 (IGSF2, V7)	?	+/?	Correlated with functional suppressor activity of CD4+ CD25+ Treg cells in mice	Fernandez et al., 2007
FR4	+	?	Plays an important role in the maintenance of Treg phenotype	Tian et al., 2012
CD26 (Dipeptidyl peptidase-4 (DPP4)	?	+/?	Plays a major role in glucose metabolism. It is responsible for the degradation of incretins such as GLP-1	Barnett, 2006

Accumulating evidence suggests that nTregs express some molecules constitutively and other molecules upon activation. They appear to constitutively express a variety of molecules, such as CD25 (interleukin-2 receptor α-chain, IL-2Rα) at a high level, CD62L, CD28, CD127, CD103, CD152 (cytotoxic T-lymphocyte-associated antigen 4, CTLA-4), and GITR (glucocorticoid-induced tumor necrosis factor receptor, also known as TNFRSF18) (Harding et al., 1992; Walunas et al., 1994; Robbins et al., 1998; Agace et al., 2000; Janeway et al., 2001; Shimizu et al., 2002; Seddon and Zamoyska, 2002). Other cell surface receptor molecules include galectin-1, CD38, CD252 (OX-40L), TNFR2, and TGF-βR1. nTregs also express high levels of CD5, L-selectin, and CD45RO, while expressing low levels of CD45RC (Piccirillo and Thornton, 2004; Mills, 2004).There is a great interest in identifying cell surface markers that are uniquely and specifically expressed on all Foxp3-expressing regulatory T cells.

Some properties of nTregs have been well documented, whereas others need more investigations. It has been shown *in vitro* that nTregs are anergic in the presence of conventional T cell stimuli such as plate-bound anti-CD3. Anergy can be prevented by additional important stimuli, for instance, IL-2 and IL-15, which can also abolish suppressive properties of nTreg cells (Koenen et al., 2003; Fehervari and Sakaguchi, 2004).

nTregs represent about 5–10% of the peripheral CD4+ T cell population (Jonuleit et al., 2001; Shevach, 2001; Sakaguchi, 2004). However, in human blood this population consists of hardly distinguishable mixture of nTregs and iTregs, and thus some authors estimate the content of nTregs at 1–2% (Ng et al., 2001). Furthermore, *in vitro* studies have shown that CD4+CD25^{high} T cells appear to be a homogeneous population of suppressor cells that do not contain memory or activated T cells. The suppressive effect is mediated by a direct cell-cell interaction–dependent, cytokine-independent mechanism that requires activation of these cells via a T-cell receptor (Nakamura et al., 2001; Jonuleit et al., 2002; Thornton et al., 2010).

Although nTregs are perhaps more abundant in a steady state of immune system, iTregs may play an important role in maintaining proper immune function and regulation (Bluestone and Tang, 2005). While nTregs need co-stimulation via CD28, iTregs do not need any co-stimulation (Kretschmer et al., 2005).

Many investigators have shed a light on nTregs for their important role in maintaining homeostasis of immune system, tolerance to self-antigens, and suppressing excessive

response to pathogens. nTregs can suppress a wide spectrum of innate as well as adaptive immune cells, including CD4+ T cells, CD8+ T cells, B cells, monocytes, NK and NKT cells, and DCs (Cosmi et al., 2003; Fehervari and Sakaguchi, 2004; Grossman et al., 2004; Piccirillo, 2004; Mills, 2004; Bruder et al., 2004; Huang et al., 2004; Sakaguchi et al., 2008). nTregs actively suppress the proliferation and cytokine production of conventional CD4+ T and CD8+ T lymphocytes. They act at an early stage of autoimmune gastritis progression to inhibit the differentiation of naive T CD4+ cells to pathogenic T helper 1 (Th1) effectors (Shevach et al., 2006), or into T helper 2 (Th2) (Xu et al., 2003) and suppress their immune response preventing from autoimmunity (Stummvoll et al., 2008). Recent evidence indicates that the use of Tregs (CD4+Foxp3+) is one of the promising approaches to control Graft-versus-Host Disease (GvHD) in different mouse models (Cohen et al., 2002; Hoffmann et al., 2002; Edinger et al., 2003; Trenado et al., 2003; Taylor et al., 2004; Ermann et al., 2005).

Tregs have shown a promising potential in therapy of autoimmune diseases and organ transplantation (Albert et al., 2006). Recently, it was found that Tregs exert an inhibitory effect on Th17 differentiation and IL-17 production, similarly to the effect on Th1 and Th2 differentiation (Chaudhry et al., 2009). However, some reports indicate that Th17 cells are not inhibited but rather enhanced by Tregs (Lohr et al., 2006; Veldhoen et al., 2006). Investigations of the role of Tregs on IL-17 production in the absence of exogenous polarizing cytokines showed that simultaneous activation of naïve conventional T cells and Tregs in the presence of antigen-presenting cells induced differentiation of Tregs into IL-17 producers (Li et al., 2010), but not conventional T cells.

1.2.2. Mechanisms of suppression

Significant progress has been made, since Sakaguchi and his co-workers published their landmark results in 1995, in delineating the molecules and mechanisms used by Tregs to mediate suppression (Sakaguchi et al., 1995). It has been accepted that Tregs are activated by either antigen-specific TCR ligation or the presence of polyclonal stimuli (Takahashi et al., 1998; Thornton and Shevach, 1998). However, after activation Tregs are able to exert their inhibitory functions in a non-antigen specific manner (Takahashi et al., 1998; Thornton and Shevach, 2000). Cell-to-cell contact between the suppressor and target cells is required for nTreg-mediated suppression (Nakamura et al., 2001; Jonuleit et al., 2002), as was shown in

experiments utilizing semipermeable membrane to separate nTregs from T effector cells; or transfer of nTreg supernatants onto effector cells. In both cases the suppression of T effectors did not occur (Ng et al., 2001). However, is still not clear which population, Th cells or APC cells, is controlled by nTregs (Shevach et al., 2001).

The presence of Treg cells is associated with decreased proliferation of T effector cells (Sakaguchi et al., 1995; Ng et al., 2001; Baecher-Allan et al., 2001), their cytokine production (Oberle et al., 2007), and altered chemokine receptor expression (Lee et al., 2004; Sarween et al., 2004). In addition to *in vitro* findings, Tregs have been shown to inhibit the expansion of T effectors *in vivo* (Edinger et al., 2003). It was found that alloantigen expression by the host APCs is necessary and sufficient for protection of GvHD by donor Tregs (Tawara et al., 2010). Inhibition of IL-2 transcription in the target cells seemed to be the main mechanism of suppression (Thornton and Shevach, 2000). Although the down-regulation of IL-2 is often used as a readout of Treg function, it is not clear if IL-2 is the primary molecular target of suppression (Sojka et al., 2008; Wang et al., 2010). Because of the indiscriminate nature of Treg suppression, the important antitumor immune response may also be suppressed, which may allow the progression of various types of tumors. Therefore, immunologists have been working to define Treg cells at the molecular level (Sakaguchi et al., 2007; Costantino et al., 2008; Agarwal et al., 2014).

Tregs use different mechanisms to exert their suppressive potential against target cells. It is not known what determines the use of a specific mechanism of suppression.

● Inhibitory cytokines

It is considered that cytokines, namely IL-10, TGF-β, and IL-35 are used by different Tregs for suppressing target cells (Fig. 1.2). In contrast to nTregs, most iTregs mediate suppression predominantly via cytokine-dependent pathways (Berthelot and Maugars, 2004). Functionally, IL-10 and TGF-β can induce peripheral iTregs (Tr1 and Th3 respectively, see Fig. 1.2). In addition, TGF-β plays an important role in the induction of Foxp3+Tregs both *in vivo* and *in vitro* (Chen et al., 2003; Liu et al., 2008) and plays a role in Tregs homeostasis (Marie et al., 2005). Activation of naive T cells in the presence of TGF-β induced Foxp3 expression and differentiation of iTreg cells (Chen et al., 2003; Zheng et al., 2004; Kretschmer et al., 2005). TGF-β1 was shown to induce IL-10 production in Th1 cells (Cottrez and Groux, 2001), which further inhibited cytokine production and directly attenuated T effector cell function

(Annacker et al., 2001). IL-10 enhanced the response of activated T cells to TGF-β1 through regulation of TGF receptor expression (Cottrez and Groux, 2001).

Some types of iTregs exert suppressive function by producing inhibitory-cytokines such as IL-10. In this mechanism Tregs can suppress differentiation of Th1 and Th2 cells directly by reducing IL-2, TNF-α, and IL-5 production, and indirectly by down-regulation of MHC and co-stimulatory molecules on APC, thereby reducing T cell activation. The mechanism of suppression will most likely depend on the type of Treg, the strength of stimuli, the nature of the immune response, and the site of inflammation (Vignali et al., 2008). However, the effects of IL-10 as immunosuppressive cytokine have been well documented in different diseases (McGeachy and Anderton, 2005; Belkaid, 2007; Rubtsov et al., 2008). IL-10 plays an important role in Treg-mediated suppression of intestinal inflammation, since blocking IL-10 or using IL-10 deficient Tregs abrogates the protective effect of Tregs on T cell transfer-induced colitis (Asseman et al., 1999).

TGF-β is a regulatory cytokine with pleiotropic functions in control of T cell responses (Li and Flavell, 2008). Generally, three members of TGF-β family have been identified, which designated as TGF-β1, TGF-β2, and TGF-β3. In early studies they were defined as negative regulators of T cells, since the addition of TGF-β to cultures inhibited T cell proliferation (Kehrl et al., 1986). Recent results have shown that co-culture of nTregs with T helper cells resulted in the differentiation of Th cells into Treg cells suppressing Th1 or Th2 cell responses in a TGF-β1 and/or IL-10-dependent manner *in vitro* (Jonuleit et al., 2002). In addition, TGF-β can exist in two forms: soluble and membrane-bound (Nakamura et al., 2001). Probably the membrane-bound form of TGF-β mediates inhibitory signals during direct interaction between nTregs with target cells (von Boehmer, 2005). Tumor suppressing activity of TGF-β is directed toward activated macrophages, T and B lymphocytes, and NK cells. Similarly to IL-10, TGF-β inhibits the expression of the MHC II on antigen presenting cells. Mode of action of TGF-β depends on the concentration of IL-2: naïve CD4+ T cells cultured with high concentration of IL-2 up-regulate CD25, IL-2 receptor beta-chain (CD122), and CTLA-4 that results in the activation and differentiation of these cells into iTreg cells (Zheng et al., 2004).

IL-35 is a newly discovered inhibitory cytokine, which may contribute substantially to the function of Treg cells directly acting on responder T cells and inhibiting their proliferation (Collison et al., 2007). IL-35 is a member of the IL-12 heterodimeric

cytokine family and constitutes a heterodimer between Epstein-Barr virus-induced gene 3 (Ebi3, which normally pairs with p28 to form IL-27) and Il12a (known as p35 that normally pairs with p40 to form IL-12 p70). IL-35 is a potent inhibitory cytokine produced by Tregs that suppresses T cell proliferation by inducing cell-cycle arrest in G1 phase without induction of apoptosis (Cottrez and Groux, 2001; Casella et al., 2014). However, unlike mouse Tregs, human Tregs do not show any tendency to constitutive expression of IL-35 (Bardel et al., 2008). Recently, it was indicated that treatment of murine or human naïve T cells with IL-35 induced iTregs-35 that can exert suppressive activity via IL-35 (Collison et al., 2010).

● Cytotoxic effects by induction of apoptosis

Targets for the cytotoxic effects include CD4+ T cells, CD8+ T cells, B cells, monocytes, and DCs. However, as mentioned above, many studies have demonstrated that mechanisms used by nTregs to suppress responder cells *in vitro* require cell-to-cell contact (Shevach et al., 2001). Thus, nTregs can exert cytotoxic effect on target cells via release of perforin and granzyme (Likuni et al., 2009), or via Fas/FasL interaction (Hirohata, 1997) (Fig. 1.2). Fas (CD95) is a type I membrane protein in the tumor necrosis factor receptor family (Nagata and Golstein, 1998). FasL (CD95L) is a transmembrane protein, and soluble FasL trimmers can be generated by a metalloproteases (Tanaka et al., 1998). The cytotoxic effects of nTregs against effector T cells have been well documented, whereas the mechanism of nTreg cytotoxic effect upon B cells was less studied and needs further investigations.

The requirement for cell-cell contact for nTreg suppressive activity have been investigated using transwell experiment model allowing to segregate between nTregs and effector T cells. It has revealed that murine Treg cells can induce cytotoxic effect toward responder T cells by granzyme and/or perforin-dependent mechanism (Gondek et al., 2005). The main function of Fas is to transduce pro-apoptotic signals from cells that express FasL (regulatory T cells) to cells that express Fas (target cells). The induction of apoptosis is therefore involved in self-tolerance by elimination of unwanted cells such as autoreactive-T and B cells.

Other results from transgenic mouse models have suggested that CD4+ T cells may play an important role in the elimination of peripheral autoreactive B cells through MHC class II/T cell receptor, CD40/CD40L, and Fas/FasL interactions (Rathmell et al., 1995; Rathmell et al., 1996). In addition to perforin/granzyme and Fas/FasL manner, nTregs use other factors that are potentially involved in killing the target

cells. These molecules include LAG-3 (Huang, 2004), membrane-bound TGFβ (Nakamura et al., 2001), cytotoxic T-lymphocyte antigen 4 (CTLA-4) (Tang et al., 2004; Read et al., 2006), galectin-1 (Garin et al., 2007), and programmed death-1 (PD-1) (Kornete and Piccirillo, 2011). All these molecules provide negative co-stimulation to target cells.

● Metabolic disruption

IL-2 is a critical cytokine in promoting survival and proliferation of T cells, by mediating differentiation and maturation of T cells into effector and memory T cells upon antigen activation of naïve T cells. Furthermore, IL-2 critically regulates several populations of mature peripheral Treg and antigen-activated conventional T cells. Additionally, in the thymus, IL-2 prevents autoimmune diseases by promoting the differentiation of certain immature thymocytes into natural regulatory T cells, which eliminates T cells that are primed to attack healthy tissues in the body (Liao et al., 2011). Its expression and secretion is tightly regulated.

In addition to the role of IL-2 in the development of T cell immunologic memory, which depends upon the expansion of the number of antigen-selected T cell clones, it also plays a key role in enduring cell-mediated immunity (Malek and Castro, 2010; Liao et al., 2011). Functionally, nTregs show a marked hypoproliferation upon TCR/CD3-mediated stimulation (Sakaguchi et al., 1995; Ng et al., 2001), which can be overcome by the addition of high doses of IL-2 (Thornton and Shevach, 1998).

Several types of Tregs are described as CD25+ and it is suggested that nTregs express high level of this molecule. This property gives the advantage to Tregs in the competition with effector T cells for binding with IL-2, that is important in maintaining homeostasis by nTreg *in vivo* (Yu et al., 2009) and crucial for their suppressive activity *in vitro* (Thornton et al., 2004). In addition, nTregs can also deplete IL-2 through binding to their constitutively highly expressed IL-2 receptor and subsequent internalization. Consequently, effector T cells, which are deprived from IL-2 by this mechanism, undergo apoptosis after 72 h of co-culture (Pandiyan et al., 2007) (Fig. 1.2). According to some authors, nTreg cells can suppress effector T cells through the inactivation of IL-2 transcription (Thornton and Shevach, 1998; Oberle et al., 2007). Other studies in the hybrid system have shown that human regulatory T cells can effectively inhibit the proliferation of effector T cells; however, the addition of human anti-CD25 antibodies blocking and binding IL-2 did not affect

the function of human regulatory T cells (Tran et al., 2009; Yu et al., 2009). Thus, the issue of participation of IL-2 as a target of suppressor cells remains controversial.

● Suppression of dendritic cells

Natural regulatory T cells can suppress T effector functions by modulating the maturation and function of dendritic cells that are required for activation of effector T cells (Bluestone and Tang, 2005). One of the molecules involved in this process is CTLA-4, an inhibitory molecule highly expressed by nTregs (Takahashi et al., 1998; Read et al., 2000). The results show that CTLA-4-deficient mice develop autoimmune diseases similar to those formed in the absence of T regulatory cells (Wing et al., 2008). CTLA-4 may participate in the suppressive activity of nTregs in two ways (Fig. 1.2). CTLA-4 interaction with co-stimulatory molecules (CD80/CD86) on dendritic cells results in inhibition or reduction of their expression. Reduced expression of CD80 and CD86 molecules on APCs weakens the co-stimulatory signal needed for the effector T cell, thereby suppressing their activation (Onishi et al., 2008). It has also been suggested that the engagement of CD80 and, to a lesser extent, CD86 on the responder T cells was responsible for the negative signal given by Treg cells (Paust et al., 2004). In addition, CTLA-4 interaction with the same molecules on DCs triggers the induction of indoleamine 2,3-dioxygenase (IDO) by DC, which catalyzes the tryptophan conversion into kynurenine, molecules showing immunosuppressive effects on several cell types , including effector T cells (Fallarino et al., 2003; Mellor and Munn, 2004).

Another molecule involves in the suppression of dendritic cells is LAG-3. It is a homolog of CD4 that binds with high affinity MHC II on immature dendritic cells. The binding of LAG-3 to MHC II induced the inhibitory intracellular signaling pathway through ITAM motifs, and resulted in inhibition of dendritic cell maturation and their ability to activate effector T lymphocyte via FcγRγ (Liang et al., 2008). Neuropilin-1 (NRP-1) can also modulate suppressor activity of nTregs. NRP-1 contributes to elongation of interaction between nTreg and dendritic cell, which restricts access of effector T cells to effective antigen presentation (Sarris et al., 2008).

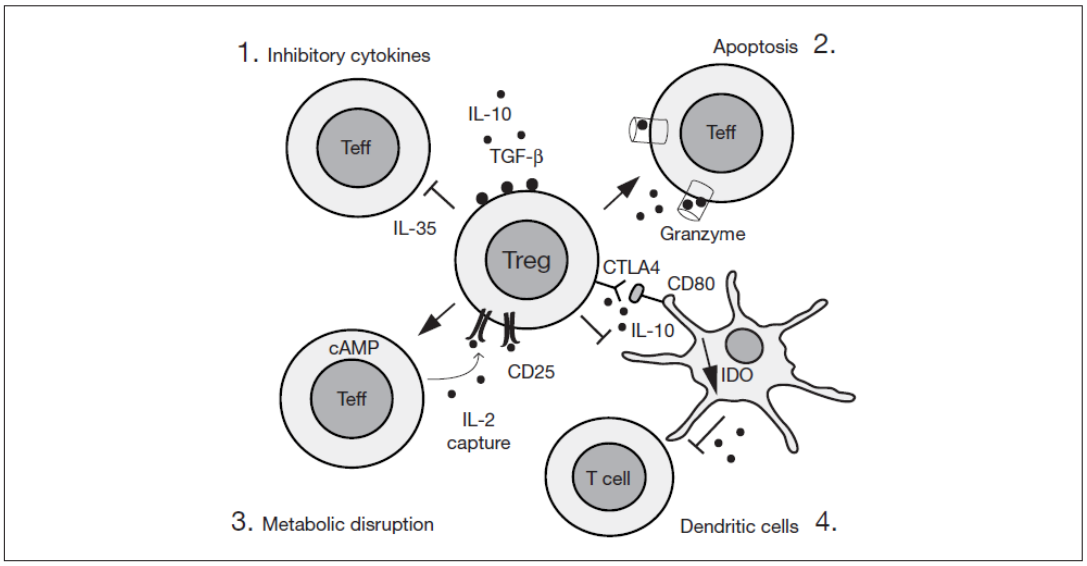


Figure 1.2:— Treg-mediated suppressive mechanisms.
1/ Inhibitory cytokines, IL-10, TGF-β, and IL-35 can suppress T cell activation. 2/ Induction of apoptosis of target effector T cells in a granzyme A- and B-dependent fashion. 3/ Cytokine deprivation, through binding of IL-2, leads to metabolic disruption of target cells or direct cAMP mediated inhibition. 4/ DC are targeted via direct cell-cell interactions, via CTLA-4 or via suppressive cytokines such as IL-10 (Broere et al., 2011).

1.3. Thymus-derived regulatory T cell development in the thymus

Jacques Miller in 1961 has indicated the thymus as the place for lymphocyte development (Miller, 1961; 2002). After one year of this landmark discovery, he described the role of thymus as a place of immunological tolerance (Miller, 1963).

Natural regulatory T cells arise in the thymus through sequential steps of thymopoiesis. It is constitutively express Foxp3 transcription factor, and these cells are first detected at the thymocyte CD4+ single-positive (SP) stage (Hori et al., 2003). Studies on Foxp3 protein expression at the single cell level in humans have confirmed that a small proportion of Foxp3+ cells are CD8+ and that Foxp3 expression is also present in a minority of CD25– T cells (Roncador et al., 2005). However, a few Foxp3+ thymocytes are also detected at the double positive (DP) CD4+CD8+ stage but most of these cells are artifacts caused by the formation of doublets of CD4+CD8+Foxp3– and CD4+Foxp3+ cells in FACS analysis (Lee and Hsieh, 2009). Nevertheless, Foxp3+ cells were also found in both DP and SP CD8+ populations.

Positive and negative selection represent two crucial checkpoints that occur during thymocyte development. At this point in development, randomly rearranged TCRs act in concert with CD4 and CD8 co-receptors to interact preferentially with either MHC class II or MHC class I, respectively (Kaye et al., 1989; Scott et al., 1989). This

engagement of TCR/co-receptor with MHC-self peptide complexes in positive selection is vital in generating T cells that can bind to self-MHC ensuring MHC restriction. In addition to that, negative selection allows elimination of potentially autoreactive thymocytes and in consequence the state of T tolerance is assured. A landmark study of thymocyte selection showed that over 97% of all thymocytes that become DP cells die because of their inability to recognize, at any level, the presence of MHC and therefore will not receive survival signals through the TCR (Huesmann et al., 1991). These cells are said to die by neglect. The remaining thymocytes are thought to survive initially by the recognition of low-affinity TCR-MHC-self peptide complexes interactions.

Signals that influence the development of nTregs *in vivo* are not clear yet. On the other hand, it is known that nTreg development is affected by different factors such as co-stimulatory molecules, cytokines, and TCR affinity. The most proximal precursor to nTreg cells is a CD4+CD25+Foxp3– thymocyte that under the proper conditions further matures into a Foxp3+ Treg cell that is able to exert suppressive function (Burchill et al., 2008; Lio and Hsieh, 2008).

Thus, a two-step model for nTreg differentiation has been proposed in which TCR/CD28 signals first induce the differentiation of nTreg precursors with increased sensitivity to IL-2/IL-15, then induce the expression of Foxp3 by IL-2/IL-15 signaling in a STAT5-dependent TCR-independent manner (Fig. 1.3) (Burchill et al., 2008; Lio and Hsieh, 2008; Goldstein et al., 2013). nTregs represent a minor population of thymocytes, i.e. about 4% of the SP CD4+ thymocytes (Fontenot et al., 2005), and between 5-10% of CD4+ in the periphery (for CD4+CD25+Foxp3+ cells including nTregs and iTregs) (Shevach, 2002; Sakaguchi, 2004).

There are two different mechanisms of T cell tolerance: central tolerance representing the clonal deletion or receptor editing of developing T cells in the thymus before they export to the periphery and peripheral tolerance related to the suppression of autoreactive T cells that escaped central tolerance and circulate in the periphery (Palmer, 2003; Stockinger, 1999). Both central and peripheral tolerance of T cells are crucial in preventing the onset of autoimmunity. nTreg cells are generally considered a key mediator of peripheral tolerance, as they are able to suppress self-reactive T and B cells and efficiently prevent autoimmunity (Piccirillo and Shevach, 2004; Fields et al., 2005). As nTregs originate in the thymus, it is thought they initially undergo the

same selective pressure and developmental checkpoints as conventional T αβ cells (Bettini and Vignali, 2010).

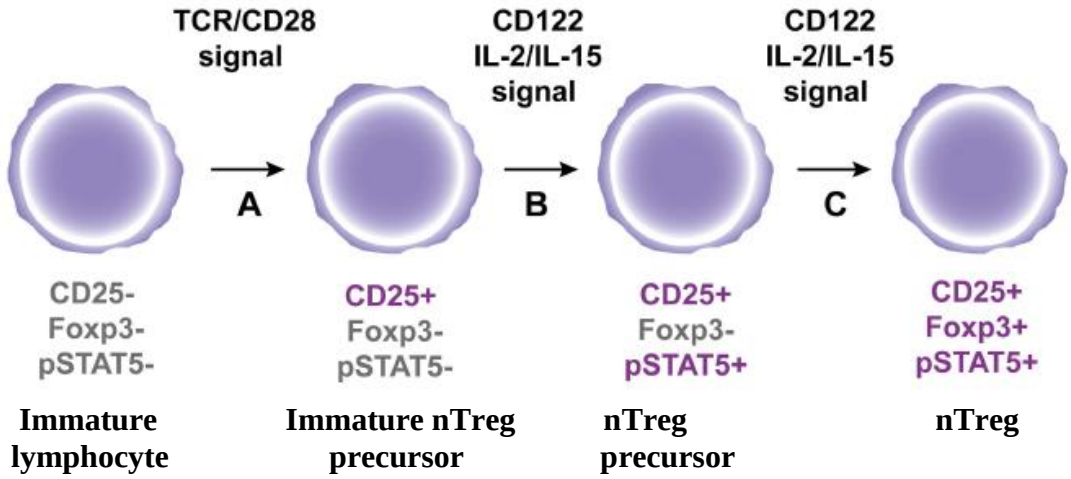


Figure 1.3:— A hypothetical model for nTreg development in the thymus.
A/ Immature CD4+ thymocytes are engaged by strong agonist TCR/co-stimulatory signals, which results in the expression of CD25. B/ Subsequent interaction of immature nTreg precursors with CD122 signaling cytokines IL-2 and IL-15 leads to STAT5 phosphorylation to generate nTreg precursors. C/ Following continuous engagement of CD122, Foxp3 expression is induced in nTreg precursors to generate fully functional nTregs (Goldstein et al., 2013).

There are two models that describe development of nTregs in the thymus. In "kinetic signaling" model (Bosselut et al., 2001) signal intensity allows for positive selection and then signal duration allows for CD4/CD8 lineage commitment. Recently, a variation of the kinetic signaling model has been proposed in which serial low affinity triggering by peptide-MHC complexes can promiscuously ligate enough TCRs to generate a persistent signal (Valitutti et al., 1995). In contradiction to this model, it has been shown that MHC class I-restricted thymocytes lacking CD8 can be driven to choose the CD8 lineage by interacting with high-avidity ligands (Goldrath et al., 1997). Furthermore, CD4+CD8$^{lo/-}$ thymocytes are not always observed within TCR transgenic mice as seen in some mice that express MHC class I specific TCRs (Hogquist, 2001). Both of these rare observations may be seen in default pathways to allow survival of thymocytes when selective pressures are applied. Regardless of the detailed events in positive selection, less than 1% of all thymocytes will recognize self-peptide at affinity/avidity high enough to receive survival signals but also low enough to avoid the activation threshold for apoptosis (Allen, 1994; Santori et al., 2002). The recognition of self-peptide with high affinity/avidity by TCR will direct the thymocyte towards controlled apoptosis and deletion (Palmer, 2003). This process is also known as negative selection. An alternative to negative selection and deletion

is the ability of these cells to undergo TCR editing and subsequently lose their autoreactive TCR and become anergic (McGargill et al., 2000). Recently, a fourth pathway has been described detailing the development of T cells selected against high affinity peptides that normally induce negative selection, sometimes referred to as agonist selection (Baldwin et al., 2004). These cell types include NKT cells, CD8αα intestinal intraepithelial lymphocytes (IEL), and possibly also CD4+CD25+ regulatory T cells and γδ T cells. It is still unclear how the intracellular signaling and genetic signatures differ between those cells selected on high versus low affinity peptides during positive selection (Bettini and Vignali, 2010). Thus, the exact events and molecules that facilitate selection of nTreg cells in the thymus are still controversial.

Accumulated evidence show that co-stimulatory molecules play a role in nTreg development. In B7 or CD28 knockout mice, or mice treated with the respective blocking antibodies markedly reduced numbers of nTregs in the thymus and in the periphery were observed (Salomon et al., 2000; Bour-Jordan et al., 2004, 2011; Tang et al., 2004). It was shown that CD28-deficient mice have approximately 80% reduction in the frequency of thymic nTreg cells (Lohr et al., 2004; Tai et al., 2005). It was observed that nTreg cell TCR repertoires of CD28-sufficient and CD28-deficient mice were not dramatically different (Lio et al., 2010). Studies using TCR-cognate antigen double-transgenic mouse models have supported these results (Hinterberger et al., 2011). Thus, it was concluded that the primary role of CD28 molecule is to enhance either the efficiency of nTreg cell development and/or the survival of thymocytes undergoing nTreg cell differentiation (Lio et al., 2010; Hinterberger et al., 2011; Hsieh et al., 2012).

It is considered that the development of nTreg cells occurs in two steps: CD4+ SP thymocytes mature into precursor nTreg cells then precursor nTregs expressing CD25 and then into functional CD4+CD25+Foxp3+ nTreg cells (Lio and Hsieh, 2008; Hsieh et al., 2012). The first step of development of nTreg depends on interactions between distinct molecules on developing nTreg with cognate molecules expressed on APC, namely: TCR/MHC II and CD28/CD80 or CD86. In consequence thymic CD4+CD25+Foxp3– nTreg cell precursors are generated. Second step is an IL-2-dependent process drives thymic nTreg cell precursors to mature CD4+CD25+ Foxp3+ nTreg cells (Fig. 1.4) (Tai et al., 2005; Lio and Hsieh, 2008; Lio et al., 2010; Klein and Jovanovic, 2011; Lu et al., 2015). In this regard, it has been shown that SP

subsets temporally preceded the development of Foxp3+CD25+ nTreg cells (Fontenot et al., 2005). In addition, it was subsequently found that the SP CD4+ subset was enriched in cells that developed into Foxp3+CD4+ T cells after intrathymic transfer (Lio and Hsieh, 2008). Notably, these nTreg cell precursors did not require further TCR stimulation to upregulate Foxp3, suggesting that nTreg cell differentiation could be divided into TCR-dependent and TCR-independent steps (Fig. 1.4) (Hsieh et al., 2012). It was suggested, however, that TCR-independent step reflected merely a delay in the time between the TCR signal and the expression of Foxp3. However, short-term culture of the SP CD4+ subset *in vitro* suggested that this was not always the case, and that additional signals were required for the up-regulation of Foxp3.

Studies on the role of cytokines in the development of nTregs suggested that IL-2 is an important player in the second step of generation of nTregs. Indeed, it was shown that the presence of IL-2 and to a lesser extent of IL-15 in the medium of *in vitro* cultured Foxp3–CD25+ thymocytes, was sufficient to rapidly induce Foxp3 expression (Lio and Hsieh, 2008), potentially through the binding of signal transducer and activator of transcription 5 (STAT5) directly to the *Foxp3* locus (Zorn et al., 2007; Burchill et al., 2007; Yao et al., 2007; Bets et al., 2014). On the other hand, nTreg cells require high affinity (TCR) ligation by an agonist peptide-MHC II complex for Foxp3 induction (Jordan et al., 2001; Relland et al., 2009). It has been suggested that affinity/avidity between TCR and II MHC-peptide complex play a pivotal role in the nTreg cell development and selection. nTreg cell development may therefore depend on multiple levels of TCR–antigen interaction as elucidated in 'niche' model (Hsieh et al., 2012). Firstly, the strength of the interaction between a TCR and an antigenic peptide–MHC class II complex is commonly referred to as "affinity", and it determines the signaling from one TCR complex. Secondly, the amount of cognate antigen per thymic APC, in conjunction with the affinity of this antigen for the TCR, would affect the overall strength of TCR signaling in a single T cell. This parameter is known as the avidity of TCR stimulation. Thirdly, the number of thymic epithelial cells and dendritic cells presenting the cognate antigen and expressing co-stimulatory molecules at sufficient levels would determine, depending on affinity and avidity, the integrated TCR signal over space and time, and this may be abstractly encompassed by the notion of a nTreg cell developmental 'niche'(Hsieh et al., 2012).

It has been suggested that the developmental signal to become nTreg cells requires unique interactions between the TCR and thymic stromal cells expressing class II

MHC–self-peptide complexes (Jordan et al., 2001; Kawahata et al., 2002). Furthermore, it seems that medullary thymic epithelial cells (mTECs) which express and present specific self-antigens in context of MHC class II molecules, support Foxp3+ nTreg selection (Aschenbrenner et al, 2007). Similarly, dendritic cells (DCs) are thought to induce CD4+CD25+ nTregs in the human thymus through T cell antigen receptor (TCR)–peptide–II MHC interaction (Watanabe et al, 2005). However, the relative contributions of thymic dendritic and epithelial cells in generating and supporting the development of nTreg repertoire are not yet completely investigated. Actually, the cellular origin, and spatial and temporal importance of the signals necessary for nTreg development have not been determined yet, and, therefore, the rules governing how developing nTregs receive and process these signals are poorly understood. On the other hand, some studies have indicated that the development of nTreg requires co-stimulatory molecule (CD28) (Tang et al., 2003), high affinity self-reactive T cell receptor signals (Hsieh et al., 2006; Liston and Rudensky, 2007), and possibly IL-2 (Bayer et al., 2007). It has been shown that IL-2 administration in mice increased Treg number and their suppressive function, and protected from chronic inflammation (Tang et al., 2008; Wilson et al., 2008; Webster et al., 2009; Dinh et al., 2012).

Recent study by Goldstein and colleagues has demonstrated that a small number of nTreg precursors expressed pSTAT5 *ex vivo* in unmanipulated neonates and they proposed that this population may be the direct precursors of pSTAT5+ CD25+Foxp3+ nTregs. Albeit, not all Foxp3+ T cells express pSTAT5, suggesting either that Foxp3 expression can be maintained without continuous STAT5 phosphorylation or that differentiation of nTreg may proceed through a STAT5-independent pathway. Thus, the exact mechanism by which CD122 signaling controls the generation of nTregs remains to be determined (Goldstein et al., 2011).

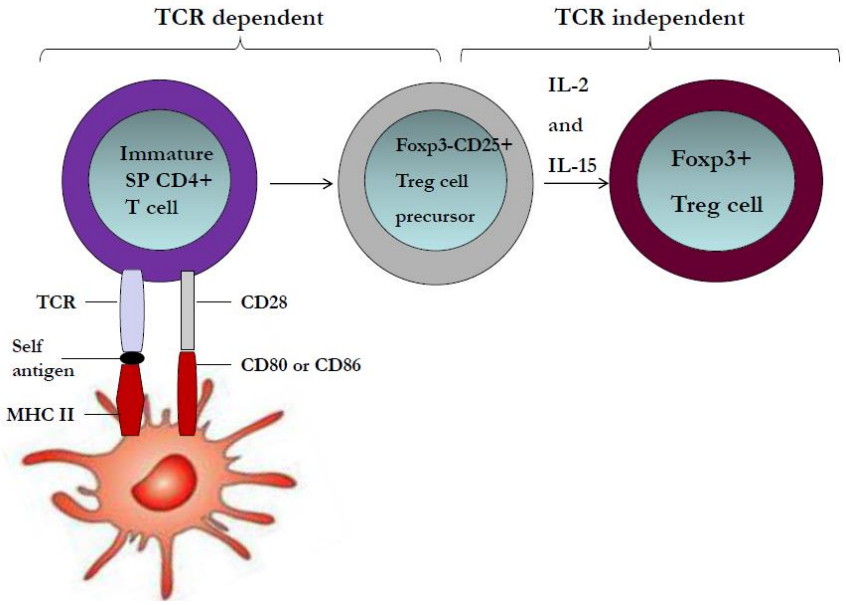


Figure 1.4:— A two-step model of nTreg cell development.
When thymocytes recognize self-peptide–MHC class II complexes in the presence of co-stimulatory molecules (such as CD80 or CD86) and with sufficiently high per cell avidity for regulatory T cell selection, some of these cells are selected as Foxp3–CD25+ nTreg cell precursors. Presumably the T cell receptor (TCR) signal leads to the activation of several downstream pathways, including nuclear factor-κB (NF-κB) activation, and results in the remodeling of the forkhead box P3 (*Foxp3*) locus, rendering it permissive to the induction of Foxp3 expression by interleukin-2 (IL-2) signaling. At this point, the nTreg cell precursor does not require further TCR stimulation for the expression of Foxp3. Instead, cytokine signals mediated by IL-2, or to a lesser extent IL-15, facilitate the induction of Foxp3 expression (according to Hsieh et al., 2012).

1.4. Role of antigen-presenting cells in the generation of Tregs

nTregs develop in the thymus and require cooperation from other cells available normally or transiently in the thymus such as thymic epithelial cells, thymic dendritic cells or other APC-like cells (Fig. 1.5 A). Several populations of DC were characterized depending on location, phenotype, and function. For instance, sirpα+ dendritic cells (DCs), CD8α+ DCs, plasmacytoid DCs, and thymic medullary epithelium have been shown to contribute to thymic nTreg development (Watanabe et al., 2005; Wan and Flavell, 2006; Aschenbrenner et al., 2007; Proietto et al., 2008; Vitali, 2009; Martin-Gayo et al., 2010; Hanabuchi et al., 2010; Cowan et al., 2013; Coquet et al., 2013). All these subsets can capture and present antigens in the lymphoid organ itself and share the capacity of antigen presentation to T cells. However, lymphoid organ-resident DCs include most of the DCs in the thymus and spleen, and half of the lymph-node DCs at the steady state.

The generation of nTregs in the thymus requires several signals which were partially described in the previous chapter. Strong signal from TCR and the interaction between key molecules, such as CD28, CD40L, GITR, and LFA-1 on T cells with

their respective ligands CD80/CD86, CD40, GITRL and CD54 (ICAM-1) on thymic stromal cells, dendritic cells, or other APCs (Malek et al., 2002; Magi et al., 2005; Jiang et al., 2006; Burchill et al., 2007; Spence and Green, 2008; Vitali, 2009).The interactions between TCR-MHC II-self peptide, CD40-CD40L and CD28-CD80/CD86 are known to be crucial in this process (Watanabe et al., 2005; Proietto et al., 2008; Spence and Green, 2008; Martin-Gayo et al., 2010; Hanabuchi et al., 2010; Coquet et al., 2013; Lu et al., 2015). For instance, in mice that lack either CD80/CD86 or CD28 a marked reduction of Foxp3+ Tregs was observed (Salomon et al., 2000; Tang et al., 2003; Tai et al., 2005; Vang et al., 2010). Furthermore, dendritic cells by capturing peripheral antigens and migrating to the thymic medulla, also play a distinct role in shaping the size and repertoire of thymic nTreg cells (Duncan et al., 2002).

Common γ-chain cytokines (i.e. IL-2, IL-15 and to a lesser degree IL-7) are required for thymic development of nTreg cells, as well as for their survival in the thymic medulla as well as in the periphery (Fig. 1.5 A).

Several specific DC subsets were found to induce CD4+CD25+Foxp3+ Tregs from activated CD4+CD25–T helpers in the periphery (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005). Due to these observations, it was assumed that DCs could be used for the *in vitro* induction of Tregs from CD4+CD25– T helpers. The induction of Foxp3 expression in naïve T CD4+ cell population occurs under suboptimal TCR signaling or by a combination of strong TCR engagement together with TGF-β or IL-2 (Vitali, 2009).

In addition to the thymus, Treg generation takes place in different conditions and locations such as in the mesenteric lymph nodes or in the gut lamina propria in the process of induction of oral tolerance or after tissue transplantation. There is a consensus in murine and human studies that the best status of DCs which can induce Tregs is tolerogenic DC. Generally, the tolerogenicity of DCs is governed by their co-stimulatory setup, with low levels of co-stimulatory molecules and high levels of inhibitory ligands, such as PD-L1 and ILT-3/4, favoring Treg induction (Schmetterer et al., 2012). One line of studies revealed that the transduction of human monocyte-derived DCs (mdDCs) with Foxp3 and co-culture of such DCs with naïve T cells led to the induction of Foxp3+ iTregs, which showed potent regulatory capacity in *in vitro* system (Lipscomb et al., 2010). Additionally, it was found that repeated co-culture of *ex vivo* prepared immature DC with allogenic CD4+ T cells leads to up-

regulation of CD25 on the surface of the T cells and to the acquisition of suppressive capacity. This new population of cells do not proliferate and is able to suppress CD4+ and CD8+ effector T cells proliferation (Jonuleit et al., 2000).

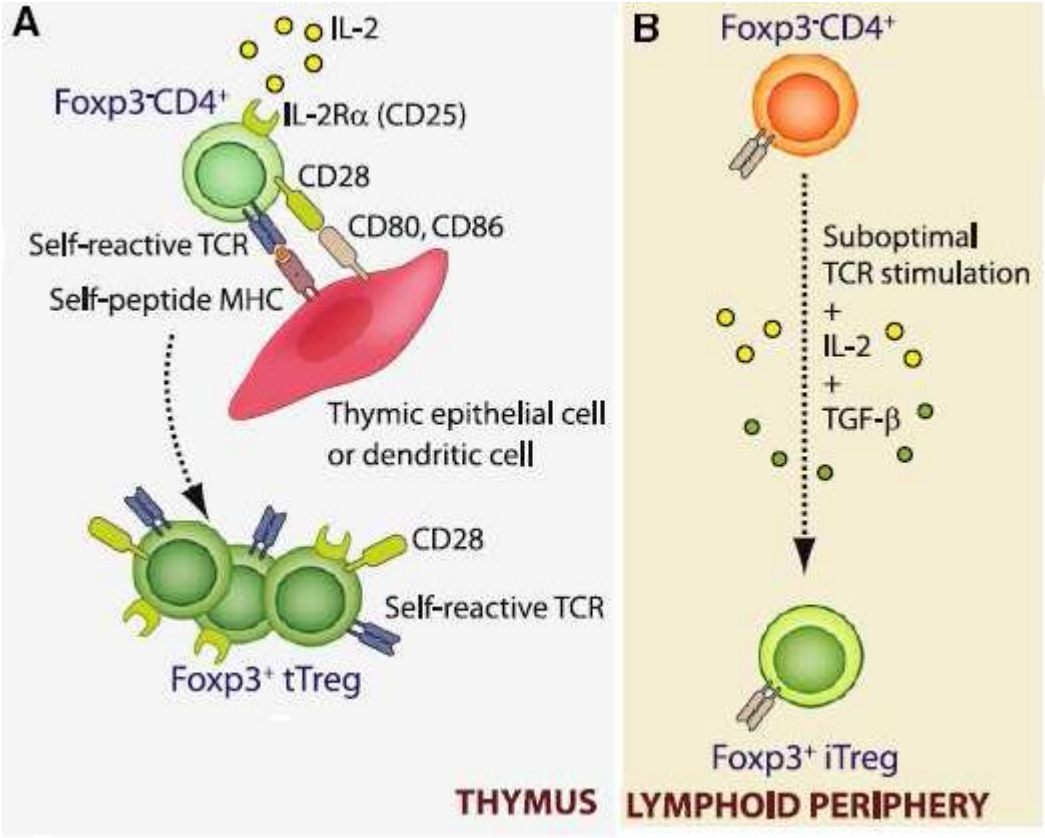


Figure 1.5:— Signals for thymic and peripheral generation of Tregs.
A/ nTreg differentiation in the thymus requires high affinity TCR engagement, CD28 binding and cytokine signaling. B/ In the periphery, under chronic low dose antigen stimulation, the presence of cytokines like TGF-β and IL-2 or other metabolites favors Treg generation (Vitali, 2009).

The role of cytokines in the generation of iTregs has been postulated. For instance, the implication of TGF-β and IL-2 in the murine iTreg conversion is contrary to the generation of nTreg for which both TGF-β and IL-2, though important, are not indispensable; the lack of TGF-β signaling is rapidly counterbalanced by higher IL-2 production (Liu et al., 2008), while IL-2 deficiency is supplemented by IL-15 (Vitali, 2009) (Fig. 1.5 B). Moreover, the endogenous TGF-β produced by CD4+CD25+ Foxp3+ Treg cells enables IL-6 to convert some of these cells to Th17 cells (Xu et al., 2007; Dons et al., 2012).

On the other hand, neutralization of IL-2 during iTreg induction abolished the development of suppressive function, while iTreg induction using allo-stimulated CD4+CD25– T cells required IL-2 and TGF-β (Zheng et al., 2007).

CTLA-4 is also involved in the generation of Tregs in the periphery as was observed in CTLA-4-deficient murine T cells that could not differentiate into iTregs. The role of TGF-β in this case is to induce or accelerate the expression of CTLA-4 during T-cell activation and in consequence induce the expression of Foxp3 after CD80 or CD86 binding. In contrast, Foxp3 expression plays a role in the up-regulation of CTLA-4 expression (Zheng et al., 2006).

Finally, the cooperation between DCs and CD4+ T cells in peripheral lymphoid organs or thymus to generate iTregs and nTregs respectively, depends on several factors such as the level of maturation of DCs or T cells, the array of cytokines available in the microenvironment, and the expression of co-stimulatory molecules on the surface of interacting cells.

1.5. Role of B cells in the induction of Tregs in the thymus and peripheral lymphoid organs

It has been found that B cells can take part in antigen presentation via MHC II molecules that are constitutively expressed on their membrane and are rapidly up-regulated upon activation (Germain and Jenkins, 2004; Byersdorfer et al., 2004; Rodriguez-Pinto and Moreno, 2005). In addition to their function as APCs, B cells exert multiple functions in adaptive immunity, for instance, antibody secretion and germinal center (GC) formation, and are critical cellular adjuvants that facilitate optimal activation of CD4+ T cell. B cells also contribute to immunoregulation due to production of cytokines including IL-4, IL-6, IL-10, IFN-γ, and TGF-β. In addition, B cells are thought to have specific roles in stimulating Ag-specific CD4+ T-cell proliferation after activation by DCs. However, their role in generation of nTregs is poorly understood as well as the immunological outcome of B cell-mediated antigen presentation has been a subject of debate, and data in this regards is limited. For instance, B cells can efficiently generate Tregs *in vitro* (Reichardt et al., 2007; Chen et al., 2009; Zheng et al., 2010; Lu et al., 2015), in contrast, they were reported to induce deletion of self-reactive T cells *in vivo* (Frommer et al., 2008). Thus, the role of B cells in the induction of T-cell tolerance to self-antigens under physiologic conditions, and in particular, in the induction of nTregs, remains to be determined.

1.5.1. Role of peripheral B cells in the induction of peripherally-derived Tregs

Direct effect of B cells on iTreg induction in peripheral lymphoid organs can be seen in *in vitro* experiments in B cells co-cultivated with naïve Treg. Splenic B cells comprise different subsets, and about 80% of them are resting, mature B2 cells (Hsueh et al., 2002). Several molecular interactions between the interacting cells are possible. By using knockout mice or receptor blockade with CTLA-4 immunoglobulin, it was shown that both CD28/B7 and CD40/CD40L interactions are important in iTreg development, as CTLA-4 depletion led to decrease in CD4+CD25+ T cell number and accelerated autoimmunity in autoimmune-prone backgrounds (Salomon et al., 2000; Kumanogoh et al., 2001). It has not been absolutely confirmed, however, whether such molecules act solely at the level of thymic nTregs generation or also affect peripheral iTregs induction, homeostasis and/or survival. Moreover, several groups have demonstrated that strong co-stimulation provided by B7 through CD28 during iTreg generation prevents Foxp3 up-regulation and renders cells with poor suppressive function (Fu et al., 2004; Benson et al., 2007; Molinero et al., 2011).

The role of other molecules in the generation of Tregs has also been studied. It has been found that OX40/OX40L, an alternative, CD28/B7-independent co-stimulatory pathway, also negatively regulates the development and function of both iTregs and nTregs. While stimulation of mature nTregs by OX40 results in the loss of suppression of T cell proliferation and cytokine production, the generation of iTregs is completely abolished by OX40, although OX40 does not affect the generation of nTregs (So and Croft, 2007).

In non-Hodgkin lymphoma mice, the number of Foxp3+ regulatory T cells is greatly increased within the tumor. This increase was critically dependent on the presence of CD70+ B cells. Additionally, removal of CD70+ B cells or blockade of the CD70 molecules, inhibited the intratumoral generation of iTreg. This result indicates that B cells, via interaction of the CD70/CD27 molecules are able to induce iTreg *in vivo* (Yang et al., 2007). On the other hand, it was documented that B cell-induced iTreg are crucial for the cure of the EAE (Experimental autoimmune encephalomyelitis). While wild type mice recover from EAE spontaneously, B cell deficient µMT mice suffer from the disease. Analysis of the affected animals revealed lack of iTreg and IL-10 in the central nervous system. After administration of wild type B cells iTreg

numbers normalized and then the animals recovered from the EAE (Mann et al., 2007). These effects were critically dependent on the expression of B7 molecules by B cells indicating that stimulation of the iTreg via CD28 pathway is critical for their induction in such conditions.

To document the impact of B cell-mediated induction of iTregs, Chen and Jensen used B cells and DCs from B6 spleens (APCs) and co-cultured them with total splenic CD4+ T cells as responder cells after depleting other type of cells. The authors have found that primary culture of BALB/c spleen CD4+ T cells contained 8–15% of Foxp3+ T cells, and after culture with B6 B cells, this percentage was increased to 31–47% in independent experiments. If DCs served as APCs this ratio was 0.08–0.19. These results indicated clearly that although B cells are less potent than DCs in allostimulation, primary B cells preferentially induced the generation of allogeneic Foxp3+ T cells (Chen and Jensen, 2007). Furthermore, in addition to their role in the generation of iTregs, recent evidence has shown that B cells are capable of expanding iTregs in the presence of co-stimulatory molecules plus IL-2 (Chen and Jensen, 2007; Chen et al., 2009). In addition, Moore and colleagues (2010) have investigated the cooperation between B cells and DCs in the generation of iTregs in the presence of TGF-β, RA (Retinoic Acid), and IL-2. Isolated B-cells were co-cultured with naïve T cells in the presence of these three factors at a 1:7 ratio of B to T cells. It was found that B cells alone were not as efficient for the generation of iTregs as partially purified CD11c enriched APCs. Additionally, isolated B cells induced higher proportion of iTregs in the absence of cytokines in comparison to partially purified APC fraction, concluding that optimal iTreg induction occurs when both populations of APCs are present (Moore et al., 2010). The mechanism by which RA enhances Foxp3 expression and increases suppressive capacity in iTreg remains controversial. Several lines of results have demonstrated that RA functions either through a cytokine-dependent mechanism, by blocking the secretion of proinflammatory cytokines by memory T cells (Hill et al., 2008), or through cytokine-independent mechanisms, because RA retains its inhibitory effect in the absence of cytokines (Nolting et al., 2009).

It was shown that B cell and F4/80^{+} ocular APC act together as inducers of Treg in the anterior chamber associated immune deviation model (Ashour and Niederkorn, 2006). In this study antigens introduced into the anterior chamber of the eye are transported by F4/80^{+} ocular APC to the spleen where the antigen is released and

picked up by B cells. Eventually, these B cells present the antigens in context of MHC II and MHC I simultaneously, giving rise to CD4+ and CD8+ Tregs respectively.

Collectively, the implication of B cells in the induction of iTregs both *in vivo* and *in vitro* needs more investigations. Also, many questions still require elucidation, such as 1/ What population of B cells and at which maturation stage can induce optimal number of nTregs? 2/ Which B cell molecules have a dominant role in this process? 3/ Are there any other factors that can facilitate and accelerate the generation of nTregs by B cells, for instance cytokines?

1.5.2. Thymic B cells and their role in the development of thymus-derived Tregs

Recently several studies have shed a light on the role of B cells in the generation of nTregs, and many of these studies has been focused on thymic B cells. B cells can play a role of APC because of their constitutive high expression of MHC II and the ability to uptake, process, and present antigens to CD4+ T cells via B cell-mediated mechanism. Additionally, B cells constitutively express co-stimulatory molecules such as CD80 and CD86 as well as CD40, and their expression increase upon activation. These molecules, as it was described for dendritic cells, have an important role in the generation of nTregs (Watanabe et al., 2005; Proietto et al., 2008; Spence and Green, 2008; Martin-Gayo et al., 2010; Hanabuchi et al., 2010; Walters et al., 2014; Lu et al., 2015).

The thymus contains low number of B cells (about 0.1–0.5% of total population of thymocytes) that were shown to contribute in thymic negative selection (Frommer and Waisman, 2010; Perera et al., 2013). Evidence that B cells can play a role in T cell selection process in the thymus has emerged from rodent studies examining minor lymphocyte-stimulating (Mls) Ags (von Boehmer and Sprent, 1974). The results have indicated that clones reactive to specific Mls Ags, such as T cells bearing the Vβ6 TCR element reactive for Mls-1a, are deleted by thymic B cells (Mazda et al., 1991; Gollob and Palmer, 1993; Tuama et al., 2000). Further studies have extended this concept to other model of Ags (Kleindienst et al., 2000; Frommer and Waisman, 2010) demonstrating that B cells can contribute to shaping of thymic T cell repertoire through negative selection.

Thymic B cells exist in the thymus during early fetal development and reach stable frequency after birth that is further maintained throughout the life (Marcos et al., 1989; Nango et al., 1991). However, the fact that B cells in the periphery have been involved in the generation of the Treg compartment (Wei et al., 2005; Zheng et al.,

2010; Morlacchi et al., 2011; Ray et al., 2012), suggested that this process may indeed be initiated firstly within the thymus. Using an *in vitro* culture model, Lu and colleagues (2015) have revealed that thymic B cells have important role in regulating the size of the nTreg compartment in the thymus. It was demonstrated that thymic B cells are localized in the thymic medulla in close proximity to nTreg precursors, particularly around Hassall's corpuscles, but also in the perivascular and intralobular spaces (Isaacson et al., 1987; Christensson et al., 1988; Walters et al., 2014; Lu et al., 2015). In addition, thymic B cells deficiency results in a significant decrease in both the frequency and number of thymic nTreg cells. Further, it has demonstrated that thymic B cells contribute to thymic nTreg cell number via cell-cell contact involving two independent pathways. In the first step, thymic B cells promote the generation of thymic nTreg precursors from CD4+ SP subsets. Then thymic B cells directly promote the proliferation of thymic nTreg cells both *in vivo* and *in vitro* that is MHC II contact dependent and an intact BCR repertoire (Walters et al., 2014) with fewer contribution of the co-stimulatory molecules CD40/CD80/CD86. Thus signals required for thymic nTreg development may be distinct from those required for thymic nTreg proliferation (Lu et al., 2015).

In addition to the above mentioned functions of thymic B cells, these cells are directly implicated in the induction of tolerance in the thymus (Ashour and Seif, 2007). In this regard, studying the mechanisms and cells responsible for the development of tolerance may improve the treatment of allergic diseases, autoimmune diseases, help to prevent transplant rejection, and the development of an immune response during gene therapy. It was suggested that B cells play a role in negative selection of CD4+CD8– SP but not CD8+CD4– SP thymocytes (Frommer and Waisman, 2010). Though activation of T cells by B cells as APC requires co-stimulation, resting B cells with low expression of B7 induce T cells tolerance (anergy). *In vivo*, dendritic cells can prevent such induced anergy by providing additional co-stimulatory signals (OX40L, cytokines) (Lohr et al., 2005; Huddleston et al., 2006).

T cell precursors expressing high-affinity receptors for MHC-self peptide complexes are eliminated. The regulator protein Aire (autoimmune regulator) plays a key role in this process. It is a transcription factor that enables the thymic cells to produce their full arsenal of endogenous proteins. Recently, it has been discovered that B cells migrating into the thymus also contribute to central tolerance. On entering the thymus, they alter their molecular profile and become more efficient in tolerance induction.

These B cells migrating into the thymus (but not peripheral B cells) also express Aire and can efficiently induce T cell-tolerance (Yamano et al., 2015a). In the same study, it was shown that Aire expression in thymic B cells coincided with MHC II and CD80 up-regulation and immunoglobulin class-switching. These features were recapitulated upon immigration of naïve peripheral B cells into the thymus, whereby this intrathymic licensing required CD40 signaling in the context of cognate interactions with autoreactive CD4+ thymocytes. Moreover, a licensing-dependent neo-antigen selectively up-regulated in immigrating B cells mediated negative selection through direct presentation (Yamano et al., 2015a).

Several studies have indicated the role of different molecules involved in the induction of T cell tolerance. However, the role of each molecule in achieving tolerance still requires further elucidation. MHC II and B7 (CD80/CD86) molecules were suggested to play an important role in tolerance development. The mechanistic studies have shown that transduced B cells must express MHC II and B7.2 (CD86) co-stimulatory molecules for successful tolerance induction (El-Amine et al., 2000; Litzinger et al., 2005). CD80 and CD86 molecules on APC interact with CD28 and CTLA-4 on T cells to transduce co-stimulatory signals (Jordan et al., 2001; Levings et al., 2005). After transient blockade of B7/CD28 co-stimulation by monoclonal antibodies prolonged allograft survival in rodent models is observed (Fowell and Mason, 1993). Other results have revealed that exposure to intra-nasal prototypic protein antigens was associated with rapid partial activation of antigen-specific B cells, characterized by increased expression of MHC II and co-stimulatory molecules. In the absence of B cells, respiratory tolerance could not be induced (Tsitoura et al., 2002).

Recently, anti-CD154 has been investigated as a new strategy for elongating allograft tolerance. In *in vivo* studies, allogeneic C57BL/6 hearts were rejected in 9 days when transplanted into untreated BALB/c recipients, while treatment with anti-CD154/DST (donor-specific transfusion) induced long-term allograft survival. This data suggested that the alloreactive-specific B cell responses are inhibited by regulatory T cells acting to constrain T cell help (Li et al., 2007; 2008). Other authors argued that anti-CD154 treatment could suppress B cell responses, but is not able to induce B cell tolerance (Foy et al., 1993). It was shown in another allograft model of tolerance induced by blockade of CD40/CD154 co-stimulation that recruitment of Foxp3+ Treg cells to

allograft tissue depends on the expression of the chemokine receptor, CCR4 (Lee et al., 2005). Studies performed in CD40L-deficient and MHC class II-deficient patients revealed that antibody reactivity of new emigrant B cells was similar to those from healthy donors, suggesting that CD40/CD40L interactions and antigen presentation do not regulate central tolerance. In contrast, mature naïve B cells from CD40L-deficient and MHC class II-deficient patients produced high percentage of auto-reactive antibodies (Herve et al., 2007).

1.6. The aim of the study

We hypothesized that B cells can be involved in the generation of thymus-derived regulatory T cells. Our hypothesis was built on the following observations. Firstly, normal development of nTreg in the thymus requires interaction with thymic antigen-presenting cells such as dendritic cells and thymic epithelial cells. These cells present self-antigens to developing nTreg allowing their selection and maturation. Secondly, very small percentage of B cells reside in the thymus, and their function and origin are still controversial. Thus, we decided to investigate if B cells, which can play a role of antigen-presenting cells for peptide antigens (Vascotto et al., 2007; Taneja et al., 2007; Roux and Niedergang, 2012), have the potential to induce the generation of thymus-derived regulatory T cells *in vitro*.

We introduced a model of co-culture of thymocytes with splenic B cells based on the results of Bieńkowska et al. (Bieńkowska et al., 2014) on the role of different sources of activatory signals necessary to the generation of thymic Tregs. Our model utilized splenic B cells as it was shown that peripheral B cells are able to migrate into the thymus (Yamano et al., 2015a). Thymocytes served as the source of nTregs precursors. We investigated the role of B cell stimulation by TLR ligands on the antigen presenting potential and its influence on nTreg generation. Lipopolysaccharide of Gram-negative bacteria (LPS) and imiquimod (IMQ) have been used as TLR4 and TLR7 ligands simulating infections by bacteria and viruses, respectively. We postulate that depending on the level of MHC II and CD80/CD86/ CD40 molecules expression, B cells can induce the differentiation of thymus-derived regulatory T cells.

To confirm this hypothesis the following issues were investigated:

1. Antigen-presenting potential of B cells activated by LPS and IMQ.

2. Potential of splenic B cells to generate nTreg cells *in vitro*.

3. Potential of thymic B cells to generate nTreg cells *in vitro* (preliminary study).

In addition, considering the influence of glucocorticosteroids on dendritic cells we have examined the role of these hormones in the induction of tolerogenic potential of activated B cells.

1.7. Study outline

1. To evaluate the antigen-presenting potential of splenic B cells the expression of MHC II, CD80, CD86, and CD40 was estimated after LPS or IMQ stimulation.

2. To examine the potential of B cells to induce the generation of natural regulatory T cells *in vitro* model of co-culture of thymocytes with splenic B cells was utilized. The model was based on two-step process of nTreg development: firstly TCR signal was delivered by B cells themselves or by anti-CD3 monoclonal antibodies, and next the co-stimulatory signal was delivered by activated B cells.

3. The suppressive function of nTregs isolated from the co-culture of thymocytes and activated B cells was evaluated in the biological assay.

4. The role of co-stimulatory molecules expressed by B cells was evaluated using blockade strategy.

5. The generation of nTregs in the co-culture of thymocytes and B cells was examined based on the distribution of the main thymocyte subsets, CD25 and Foxp3 expression.

6. The role of thymic B cells in nTreg generation was examined in cultures of thymic cells in LPS or IMQ-conditioned culture.

7. The role of glucocorticoids on nTreg generation was investigated in the co-cultures of thymocytes and activated B cells.

2. Materials and Methods

2.1. Mice

8–12 weeks old B6.Cg-Foxp3^{tm2Tch} male mice (expressing Foxp3 protein in co-expression of GFP protein), as well as 8-10 weeks old male C57BL/6 mice were used in experiments. Mice were bred and maintained in the Animal Facility at the Faculty of Biology, University of Warsaw in the individually ventilated cages system (IVC) LD (light/dark) 12/12 h, with free access to standard food and water. All the procedures involving animal studies have been approved by the Local Ethic Commission under permission number 30/WB/2010**.**

2.2. B cell purification

The mice were sacrificed in CO_2 chambers. Freshly isolated spleens were placed into cold PBS and immediately homogenized. The cells were filtered through 50 µm cell strainer (Becton-Dickinson) to remove tissue debris. The cells isolated from organs were counted and a suspension of cells in PBS at the appropriate density was prepared for further analysis. The viability of the cells was determined by staining with 0.4% trypan blue solution. B cells were obtained by depletion of T cells using anti-Thy-1 coated (CD90.1) microbeads by magnetic cell sorter (MACS, Miltenyi Biotec). Briefly, splenic cells were washed 1 time with PBS and red blood cells (RBCs) were lysed using RBC lysing solution. After RBCs lysis, the splenic cells were washed twice with PBS. B cell separation was performed according to the manufacturer's procedure. In brief, splenic cells were suspended in MACS buffer (10^7 cells/90 µl of the buffer), and 10 µl of anti-Thy-1 coated beads were added. The mixture was incubated 15 min at 4^oC. After the incubation, the cells were washed twice with MACS buffer. Finally, the cell suspension was sorted using MACS instrument according to the manufacturer's instructions. The purity of splenic B cells was > 96%, as assessed by flow cytometry by staining with anti-CD3 monoclonal antibodies conjugated with PE and anti-CD19 MoAb conjugated with APC. The purified B cells were resuspended in culture medium for all further *in vitro* cultures.

2.3. Cell culture conditions

For the *in vitro* cell cultures RPMI-1640 Glutamax medium (Gibco) supplemented with antibiotics (Penicillin 50 mU/ml and Streptomycin 50 ng/ml)

(Gibco), 10% fetal bovine serum (Gibco), 1 mM sodium pyruvate (Gibco), and 0.2 mM 2-mercaptoethanol (Gibco) was used. Thymocytes and B cells were cultured at 37ºC in a 5% CO_2 humidified atmosphere for 24 and 72 h.

To characterize the expression of MHC II and co-stimulatory molecules on B cells, splenic cells were cultured in flat-bottom 24-well plates in LPS or IMQ-conditioned medium. Non-activated splenic cells served as control.

To investigate the role of B cells in the generation of nTreg cells thymocytes were co-cultured with isolated splenic B cells. Thymocytes at the density of 2×10^6 cells/ml and B cells were co-cultured in 10:1 and 1:1 ratio in flat-bottom 24-well plates.

The following culture conditions were applied:

- Co-culture of thymocytes with non-activated and activated isolated splenic B cells was performed. LPS from *Escherichia coli* (0111:B4) (SIGMA) (1 µg/ml and 0.01 µg/ml) and imiquimod, IMQ, (SIGMA) (5 µg/ml and 1 µg/ml) were used to activate B cells. The concentrations were chosen according to (Xu et al., 2008), and (Sauder, 2000), respectively.

- The control samples consisted either from non-activated thymocytes culture alone to compare with co-cultures or non-activated splenocytes culture alone to compare with activated splenocytes.

- Co-cultures of anti-CD3 pre-activated thymocytes with non-activated and isolated activated splenic B cells were performed. The plate was coated with anti-CD3 monoclonal antibodies (BD Pharmingen) (5 µg/ml) according to (Bieńkowska et al., 2014). Next the thymocytes were cultured 24 h on anti-CD3 pre-treated plates before addition of B cells. Anti-CD3 pre-activated thymocytes served as a control.

- Co-cultures of thymocytes with activated B cells in the presence of Dex (10^{-8} to 10^{-12} M) were performed.

2.4. Flow cytometry analysis of B cell markers

To analyze the expression of MHC II and co-stimulatory molecules (CD80, CD86, CD40) on B cells, the following monoclonal antibodies were used: anti-

CD19/APC (clone 1D3), anti-CD80/PE (clone 16-10A1), anti-CD86/PE (clone GL1), anti-CD40/PE (clone 3/23), and anti-MHC II/PE (clone M5/114.15.2), all reagents have been purchased from BD Pharmingen. B cell staining was performed according to standard procedure. The gating strategy to analyze B cell markers is presented in Fig. 2.1.

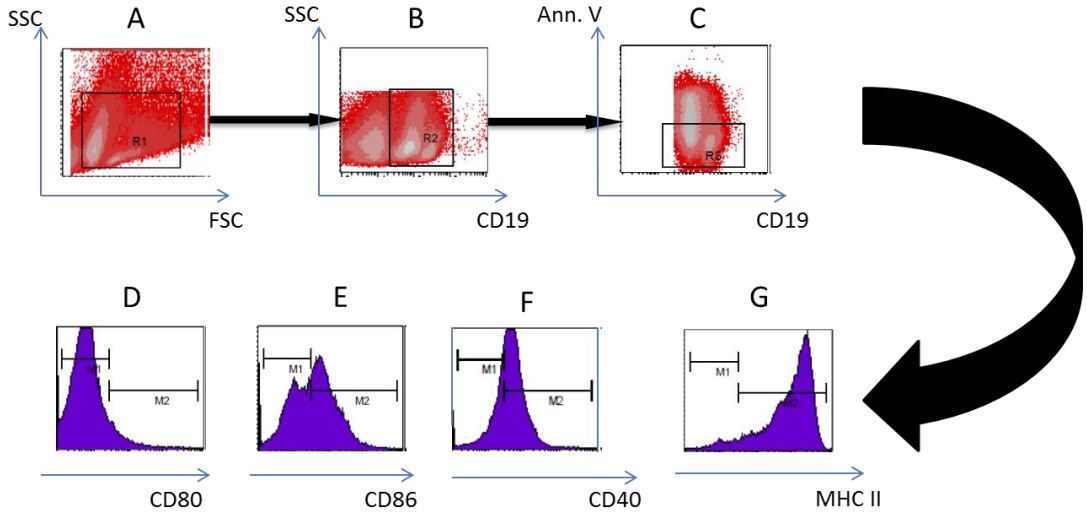


Figure 2.1:— Gating strategy of splenic B cells.

A/ Whole population of splenocytes (R1), B/ CD19+ B cells have been gated (R2), C/ CD19+ living B cells have been gated (R3). Percentage of living-splenic B cells positive for CD80 (D), CD86 (E), CD40 (F), and MHC II (G) molecules has been analyzed.

2.5. Flow cytometry analysis of thymus-derived Treg generation in the co-culture with B cells

Thymocytes are classified into four main subsets according to the expression of CD4 and CD8 molecules: CD4−CD8− (double negative: DN), CD4+CD8+ (double positive: DP), CD4+CD8−, and CD8+CD4− (single positive: SP). Natural regulatory T cells (nTregs) are recognized as CD4+Foxp3+ within SP CD4+CD8− thymocytes or as CD4+CD25+Foxp3+ in the same thymocyte subset (SP CD4+CD8–).

B cells co-cultured with thymocytes were determined basing on the expression of CD19.

For thymocyte staining the following monoclonal antibodies were used: anti-CD4/PerCP (clone RM4-5) or PE (clone H129.19), anti-CD8a/PE (clone 53-6.7) or APC (clone 53-6.7) or PerCP (clone 53-6.7), anti-CD25/APC (clone PC61) or PE-Cy^{TM}7 (clone PC61), and Foxp3/FITC (Foxp3 co-expressed with GFP protein), all reagents have been purchased from BD Pharmingen.

The staining has been performed according to standard procedure. In brief, lymphocytes were suspended in cell wash supplemented with 0.5 % BSA (bovine serum albumin) at 2×10^7 /ml density (1milion cells per 50µl). 50µl aliquots of cells suspension were transferred to FACS tubes. Suitable monoclonal antibodies conjugated with fluorochromes were added to each sample and incubated (temperature 4°C, 30 minutes, in dark). After incubation, the thymocytes were washed twice with PBS, and the expression of markers was determined based on the fluorescence emission by antibodies conjugated to fluorochromes. Flow cytometry analysis was performed on a FACSCalibur or FACSVerse (BD Bioscience). The results were analyzed using CELL Quest or FACSuite/FACSDiva. The distribution of thymocytes populations was expressed as percentages.

The gating strategy to analyze the generation of nTregs is presented in Fig. 2.2.

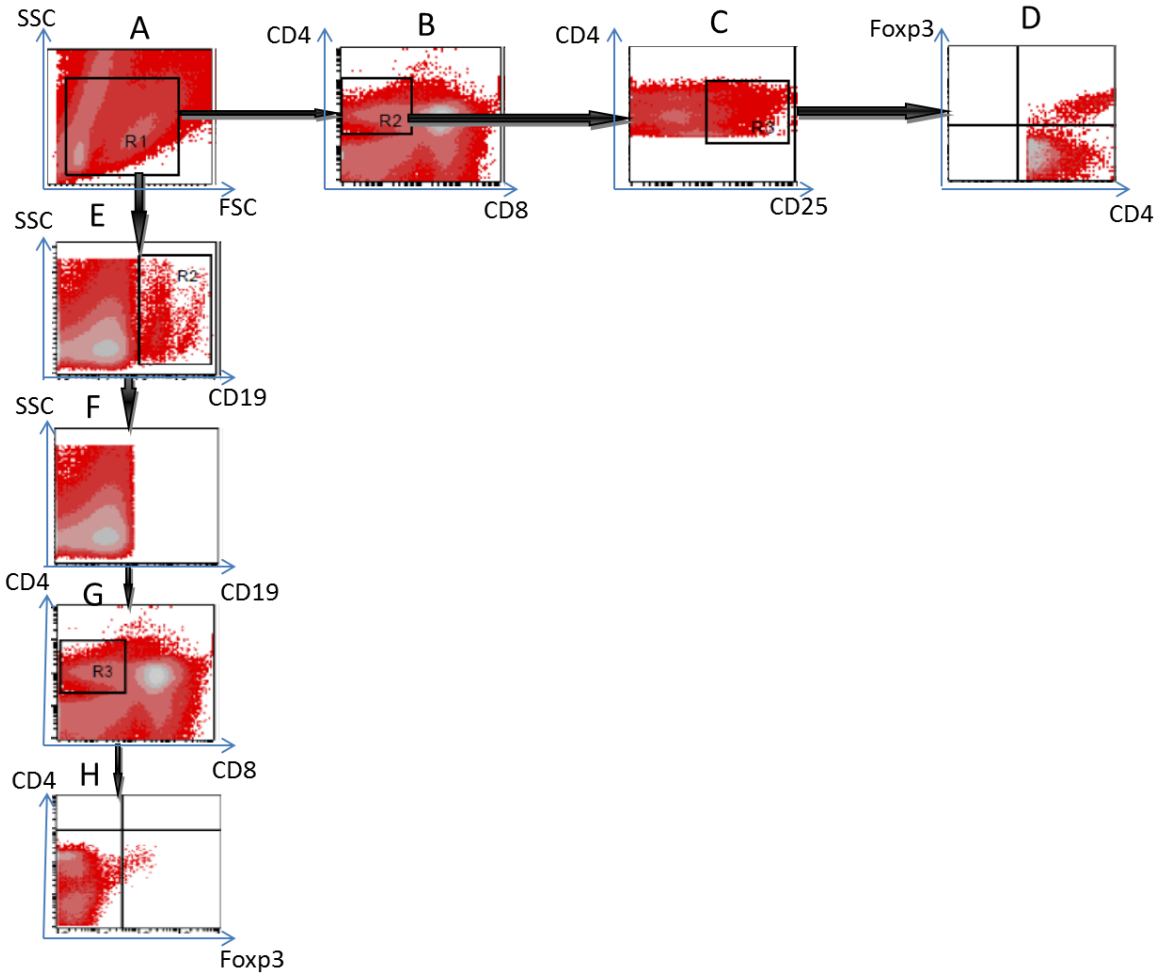


Figure 2.2:— Gating strategy and phenotype analysis of *in vitro* generated CD4+CD25+Foxp3+ nTregs in co-cultured with splenic B cells.
A/ Whole population of cells (R1), B/ The percentage of SP CD4+ thymocytes have been gated for further analysis (R2), C/ Gated SP CD4+ thymocytes have been analyzed for CD25+ expression (R3), D/ Gated CD4+CD25+ thymocytes have been analyzed for Foxp3 expression. From (E) to (H) represented gating strategy and phenotype analysis of *in vitro* generated CD4+Foxp3+ nTregs in co-cultured with splenic B cells. Whole population of cells R1 (A). To perform further analysis on nTregs only, CD19+ B cells have been excluded (R2) (E), and to be sure that B cells have been excluded from further analysis (population in R2 were excluded from further analysis) (F). SP CD4+ cells have been gated (R3) for further analysis (G). Gated CD4+ thymocytes have been analyzed for Foxp3 expression, CD4+Foxp3+ (H).

2.6. Estimation of cell viability and proliferation

The viability and proliferation of CD4+CD25+Foxp3+ (nTregs) cells in co-culture, were measured after 72 h using FACSVerse (BD Pharmingen). To determine cell proliferation Violet Proliferation Dye 450 (VPD450) was used and 7-Amino-Actinomycin D (7-AAD) was used to assess cell viability. Both dyes were purchased from BD Pharmingen.

The percentages of apoptotic DN, DP, and SP thymocytes as well as splenic B cells were measured by FACSCalibur using Annexin V/PE (BD Pharmingen). To

discriminate between living and dead splenic B cells, Annexin V/FITC was used to exclude dead cells from the analysis (Fig. 2.1 C).

The level of apoptosis was determined in the main subsets of thymocytes and the population of nTreg. Staining of the cells was carried out in two stages:

1/ Surface markers labeling using anti-CD4/PerCP, anti-CD8/PE, and anti-CD25/APC antibodies; 2/ Apoptosis analysis using Annexin V conjugated to phycoerythrin - PE (Annexin V-PE Apoptosis Detection Kit, BD Pharmingen) at a concentration of 0.5 µg in stock /10^6 thymocytes. The samples were incubated in the dark for 15 min. at room temperature. The results were analyzed by the CellQuest program on FACSCalibur cytometer.

2.7. Determination of the suppressive activity of thymus-derived Tregs in the assay of inhibition of proliferation of activated-responder T cells

Thymus-derived Tregs were sorted from 24 h co-cultures with splenic B cells. For nTreg sorting thymocytes were labeled with the following monoclonal antibodies: anti-CD4/PE, anti-CD8/PerCP, anti-CD25/PE-Cy^{TM}7, and subsequently CD4+CD25+ nTreg cells were sorted using FACSAria (Fig. 2.3).

Responder T cells were isolated from axillary lymph nodes and suspended at 2.5 × 10^5 cells/ml in complete medium (10% FBS, 2-ME, Hepes 1M, Penicillin/ Streptomycin, RPMI 1640 Glutamax-I, Gibco). Anti-CD3 (0.5 µg/ml) and anti-CD28 (0.05 µg/ml) monoclonal antibodies (BD Pharmingen) were used for responder T cell activation. Proliferation rate was measured by staining the responder T cells with the fluorescent dye CFSE (carboxyfluorescein diacetate succinimidyl ester) (Lyons, 2000; Hawkins et al., 2007; Quah et al., 2007). Labeling was performed using Cell Trace CFSE Cell Proliferation Kit (Invitrogen) according to manufacturer's instructions. In brief, suspensions of lymphocytes isolated from lymph nodes in PBS supplemented with 0.1 % BSA were prepared. Suspension density of each sample was 2 × 10^7/ml. 10µM solution of CFSE in PBS supplemented with 0.1% BSA was prepared. The same volumes of cell suspensions and CFSE solution were mixed and incubated for 10 minutes, temp. 37°C. After 1 minute cells were washed twice with medium. Cell suspension at the density 2 × 10^6 /ml was used in further experiments.

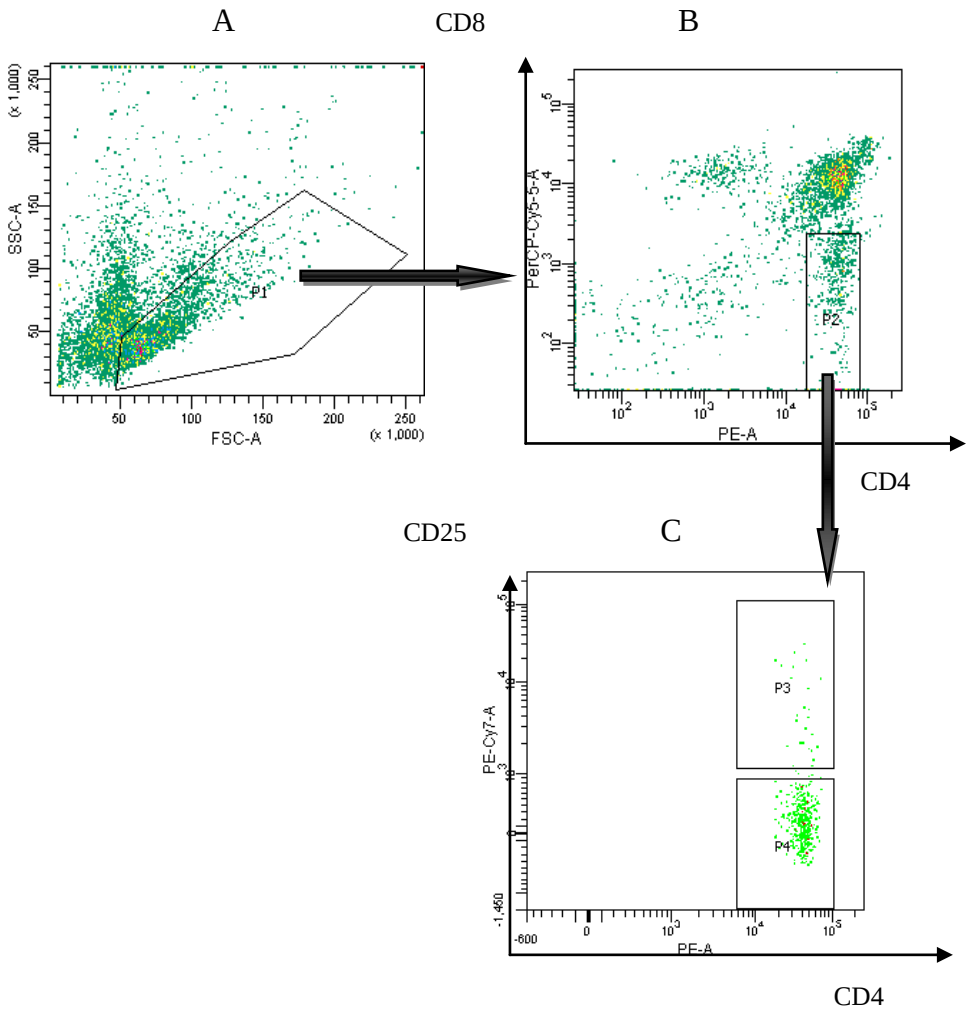


Figure 2.3:—Schematic diagram of nTreg cell sorting.
The labeled thymocytes: A/ Identified thymocyte population (P1); B/ T cell population of CD4 + (P2); C/ SP population of CD4+ thymocytes with CD25 expression (P3) was isolated; CD4+CD25− population was excluded (P4).

In order to determine the suppressive activity of nTregs isolated from 24 h co-culture of thymocytes with splenic B cells, the following types of cultures were performed:
1/ negative control – non-activated responder lymph node T cells; 2/ positive control (proliferation control) – responder T cells activated with anti-CD3 and anti-CD28 monoclonal antibodies; 3/ co-culture of responder T cells and nTregs. Responder T cells were cultured with nTregs in 5:1 ratio (25×10^3 / 5×10^3 cells/ml). The cells were cultured in 96-well round bottom plate in a final volume of 200µl and incubated at 37°C and 5% CO_2. The proliferation rate of responder T cells was analyzed using a FACSCalibur flow cytometer (BD Bioscience) after 72 h, and the analysis was performed using CellQuest software.

nTreg suppressor activity was presented as the percentage inhibition of proliferation calculated according to the following equation (Bollinger et al., 2009):

$$\% \text{ inhibition} = 100 - \frac{\% \text{ of proliferation of responder T cells with nTregs}}{\% \text{ of proliferation of responder T cells}} \times 100$$

2.8. Statistical analysis

All experiments have been repeated at least in 3 independent times. The results are expressed as means ± SD. Data were analyzed using ANOVA from SPSS program (version 23) for percentage of cells, and unpaired, Student's *t* test for MFI (Mean Fluorescent Intensity). The significance values were indicated as▲ between control (non-activated) and activated cells in 24 or 72 h cultures, ● between the concentrations of LPS or IMQ for the same time of culture, and * between 24 and 72 h cultures. The probability levels were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for other symbols).

3. Results and Discussion

The results of this dissertation were divided into three sections:-

1. Role of splenic B cells in thymus-derived regulatory T cell development.
2. Thymic B cells in the development of nTregs: preliminary studies.
3. Effect of dexamethasone on the generation of nTregs in co-cultures of thymocytes and splenic B cells.

3.1. Role of peripheral B cells in thymus-derived regulatory T cell development

B cells are antigen presenting cells and can be involved in T cell activation and development of an immune response or the induction of tolerance by the differentiation of regulatory T cells that can inhibit the immune response by various mechanisms. B cells are considered as professional antigen presenting cells due to the constitutive expression of MHC II molecules, and ability to process the antigens endocytosed after binding with BCR. The result of antigen presentation was controversial as it was shown that, under certain conditions, B cells are tolerogenic rather than immunogenic (Fillatreau et al., 2002; Raimondi et al., 2006). Upon activation via TLR ligands, B cells rapidly up-regulate molecules important in B-T interaction including MHC II, CD80, CD86, and CD40 (Xu et al., 2008; Hanten et al., 2008; Hua and Hou, 2013). In this section we focused on the evaluation of:

- the potential of LPS- and IMQ-activated B cells to present antigens as measured by the expression of MHC II and co-stimulatory molecules important in the interaction of B cells with T helper cells:
- the potential of activated B cells to induce the development of nTregs in the *in vitro* model of co-culture of thymocytes and splenic B cells:
- the possible mechanism of nTreg generation:
- the suppressive activity of nTregs generated *in vitro*.

TLRs signaling is important in B-cell activation and maturation (Isaza-Correa et al., 2014). Naïve murine B cells express a repertoire of TLRs and proliferate *in vitro* in response to TLR ligands including TLR4 and TLR7 (Gray et al., 2007; Barr et al., 2007; Hwang et al., 2009). TLR-induced signaling increase the expression of molecules involved in B-cell mediated activation and differentiation of CD4+ T cells. We analyzed the role of TLR4 and TLR7 ligands (LPS and IMQ respectively) in the activation of B cells as measured by the expression of molecules important for their

antigen-presenting potential, and we examined the potential of such activated B cells to induce the generation of nTregs according to the two-step model of nTreg development involving signals both from TCR and co-stimulatory molecules.

3.1.1. Expression of co-stimulatory molecules and MHC II on splenic B cells upon activation by LPS and IMQ

The results of our studies showed that LPS and IMQ stimulation increased the percentage of CD80 positive splenic B cells (Fig. 3.1 A). The effect depended on the LPS concentration, but did not depend on the duration of culture for the same concentration used. The increase of the percentage of CD80+ B cells induced by IMQ was not dose- or culture duration-dependent. The only exception was 1 μg/ml dose that resulted in decrease in the percentage after 72 h of culture compared to 24 h. LPS and IMQ stimulation increased the percentage of CD86+ B cells (Fig. 3.1 B).

In LPS-stimulated cultures the increase of the percentage was concentration-dependent, but culture duration-independent. IMQ stimulation-induced the increase of the percentage of CD86+ B cells which did not depend on the concentration used nor the duration of the culture. The percentage of CD40+ B cells was not changed by LPS or IMQ stimulation in 24 h culture, and was maintained at the 24 h control level after 72 h of culture (Fig. 3.1 C). LPS and IMQ stimulation did not influence the percentage of MHC II+ B cells (Fig. 3.1 D).

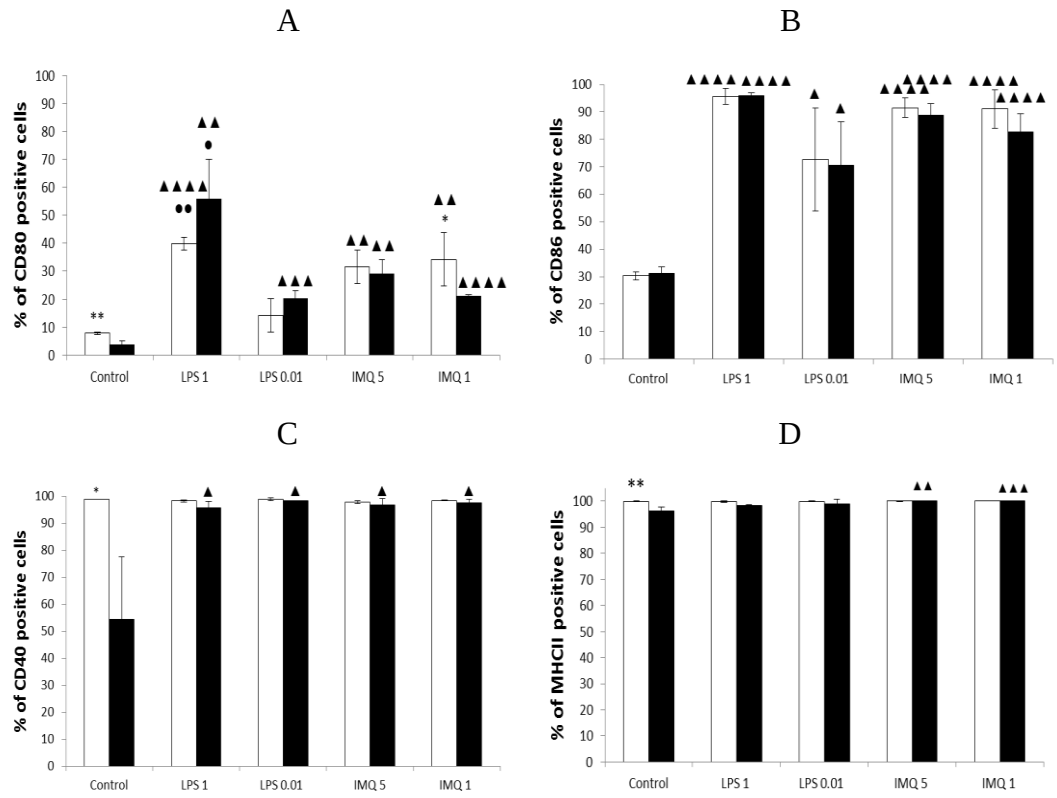


Figure 3.1:— Effect of LPS and IMQ on the percentage of B cells expressing co-stimulatory molecules.

CD80 (A), CD86 (B), CD40 (C), and MHC II (D) *in vitro*. The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as▲ between Control (non-activated) and activated B cells in 24 or 72 h cultures, ● between the concentrations of LPS or IMQ for the same time of culture, and * between 24 and 72 h cultures. Control (non-activated cells), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), IMQ5 (5µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for other symbols).

Next, the expression of CD80, CD86, CD40, and MHC II molecules on splenic B cells was investigated. Figure 3.2 shows the surface expression of CD80, CD86, CD40, and MHC II on splenic B cells in 24 and 72 h of culture. The expression of CD80 was not changed upon LPS activation, but was increased in 24 h cultures and decreased in 72 h cultures upon IMQ stimulation (however, the decrease was not statistically significant). In IMQ-stimulated cultures up-regulation of CD80 in 24 h cultures and its down-regulation in 72 h cultures were not dose-dependent (Fig. 3.2 A).

The expression of CD86 increased upon activation by LPS and IMQ compared to control, and was both concentration and duration-dependent. The expression of CD86 showed a significant increase upon activation and for all activator concentrations (Fig. 3.2 B). The expression of CD40 and MHC II increased upon activation with LPS and IMQ, and was concentration and duration-dependent for CD40 (both activators) and

MHC II (LPS only). The expression of CD40 and MHC II increased in 72 h cultures compared to 24 h cultures (after activation with IMQ 5 µg/ml) however, the increase was not statistically significant (Fig. 3.2 C and D).

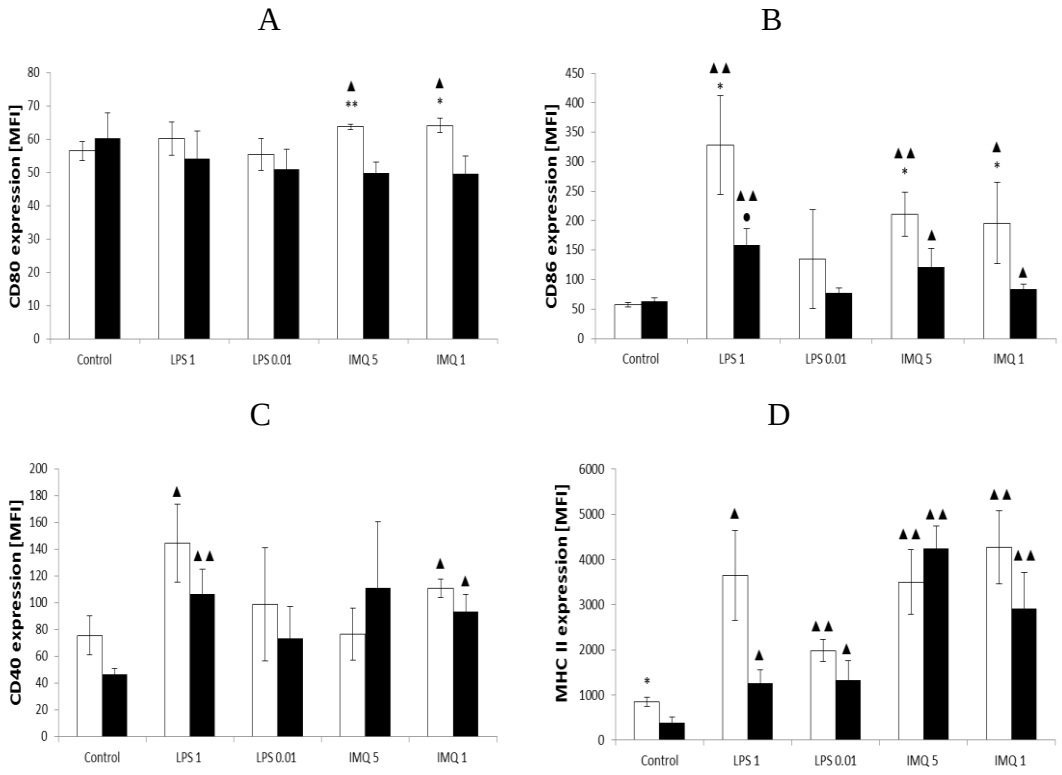


Figure 3.2:— The expression of co-stimulatory molecules.
CD80 (A); CD86 (B); CD40 (C); and MHC II (D) on LPS- and IMQ-activated B cells. The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as▲ between Control (non-activated) and activated B cells in 24 or 72 h cultures, ● between the concentrations of LPS or IMQ for the same time of culture, and * between 24 and 72 h cultures. Control (non-activated cells), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), IMQ5 (5µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001, and similarly for other symbols).

Summary:

1. The activation of B cells through TLR4 or TLR7 ligands (LPS and IMQ respectively) increased the percentage of CD80, CD86 positive B cells, counteracted the decrease of the percentage of CD40 positive B cells, and did not influence the percentage of MHC II positive B cells.
2. The activation of B cells through TLR4 or TLR7 ligands strongly increased the expression of MHC II, CD86 and CD40, with minor effect on CD80 expression.

3. All these changes showed that TLR4 and TLR7-mediated B cell activation modulate the potential of B cells to present antigens and activate CD4+ T cells.
4. We did not observe any substantial differences in the percentage of positive B cells for all of investigated markers upon activation by LPS or IMQ (with one exception for CD80 positive B cells in LPS1 activated cultures compared to both concentrations of IMQ). Similar difference has been observed for the expression of CD86.

3.1.2. Thymocyte subsets distribution in co-cultures of splenic B cells and thymocytes

To follow the role of B cells in the development of nTregs we investigated the sequential developmental steps and the effect of activation of B cells on normal distribution of thymic subsets. Additionally, we examined the effect of thymocytes : B cells ratio on the efficiency of nTreg generation *in vitro*. These experiments were performed to examine if B cells in direct contact with thymocytes are able to shift the development to CD4+ thymocytes containing also the precursors of nTregs. Thus, we have examined this in different experimental conditions: using booth non-activated and LPS- or IMQ-activated B cells co-cultured with intact (non-pre-activated thymocytes (Ctrl).

It has been documented that nTreg cells represent a minor population of thymocytes. It consists of about 4% of the SP CD4+ thymocytes (Fontenot et al., 2005), and a few Foxp3+ thymocytes are also detected in the double positive (DP) CD4+CD8+ subset (Lee and Hsieh, 2009). Accordingly, the normal distribution of thymocyte subsets (DN, DP, SP CD4+, and SP CD8+) was studied in thymocytes culture alone (Ctrl) and compared to the distribution of these subsets in the 24 h and 72 h co-cultures of thymocytes with splenic B cells to evaluate the effect of B cell activation on this process.

We excluded the high concentration of IMQ (5µg/ml) as the outcome of IMQ-mediated activation was similar for both concentrations used (Fig. 3.1 and 3.2).

The distribution of the main thymocyte subsets in the co-cultures of thymocytes with splenic B is presented in Figure 3.3. We have observed that the co-culture (thymocytes : B cells ratio 10:1) significantly increased the percentage of DN thymocytes and decreased the percentage of DP thymocytes compared to thymocytes

culture alone (Ctrl) (Fig. 3.3 A and B 24 h). These results suggest the possible block in the development of DN thymocytes to DP stage, while maintaining the control level of SP CD4+ thymocytes. The effect of splenic B cells interaction with thymocytes resulted in the significant decrease of SP CD8+ thymocytes compared to the control group (Fig. 3.3 C and D 24 h). This effect did not depend on the activation of B cells.

When the co-culture was extended to 72 h, thymocyte distribution was similar to 24 h co-culture (Fig. 3.3 A, B, and D 72 h) except SP CD4+ thymocytes. The percentage of these thymocytes decreased in the presence of non-activated and LPS1-activated B cells and reached the control level when B cells were activated by the low dose of LPS and IMQ1 (Fig. 3.3 C 72 h). The distribution of the thymocyte subsets showed the similar pattern in thymocytes : B cells ratio 1:1 (Fig. 3.4).

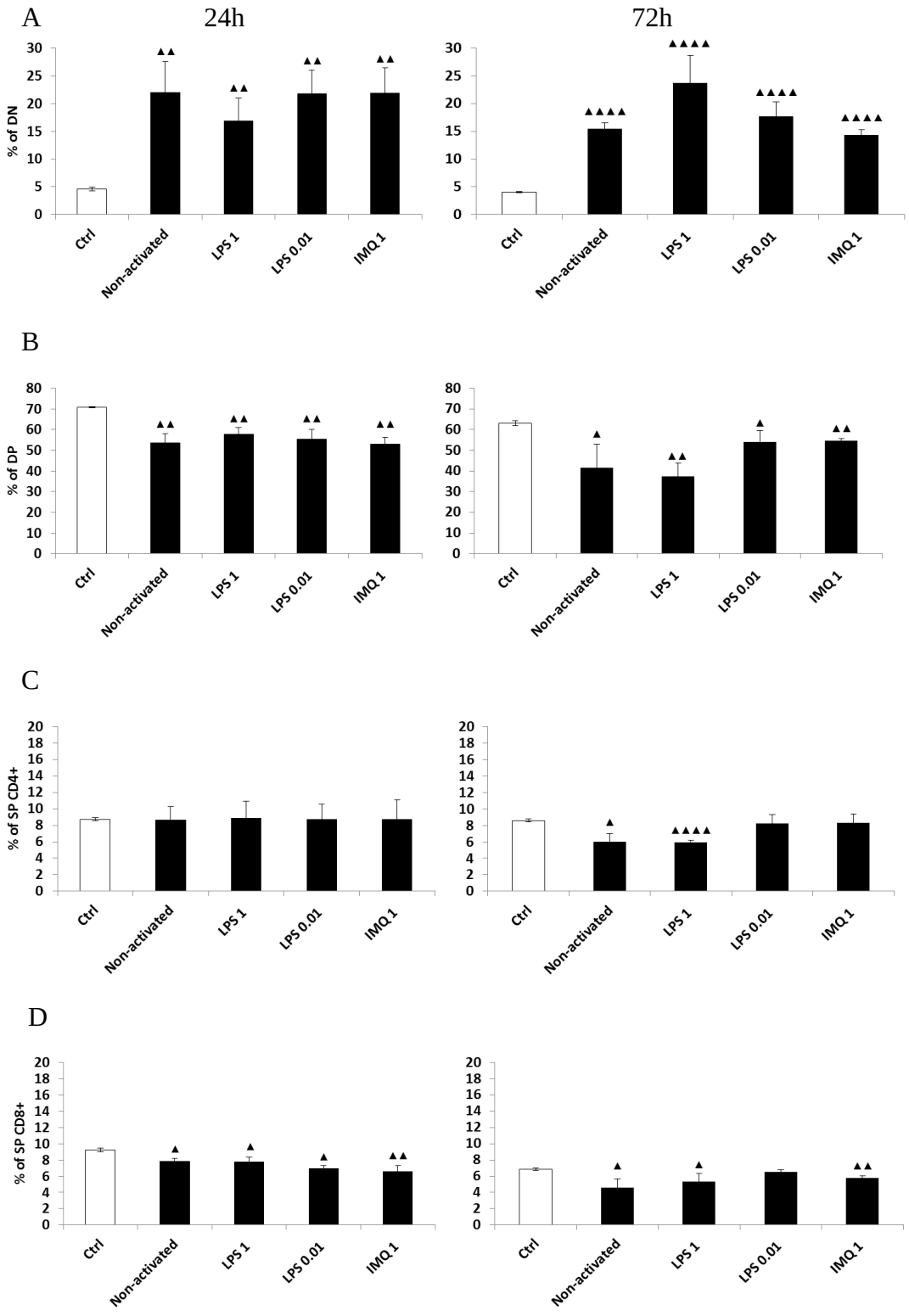


Figure 3.3:— Distribution of thymocyte subsets.
The experiments have been performed in triplicates. Thymocyte subsets distribution was analyzed after 24 and 72 h of *in vitro* culture in thymocytes culture alone (Ctrl, control non-pre-activated thymocytes) (white bars) and in co-culture with splenic B cells (black bars, thymocytes : B cells ratio 10:1). DN (A), DP (B), SP CD4+ (C), and SP CD8+ (D). The significance values were indicated as ▲ between Ctrl and co-cultures. Ctrl (non-pre-activated thymocytes), non-activated (non-activated co-culture), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$).

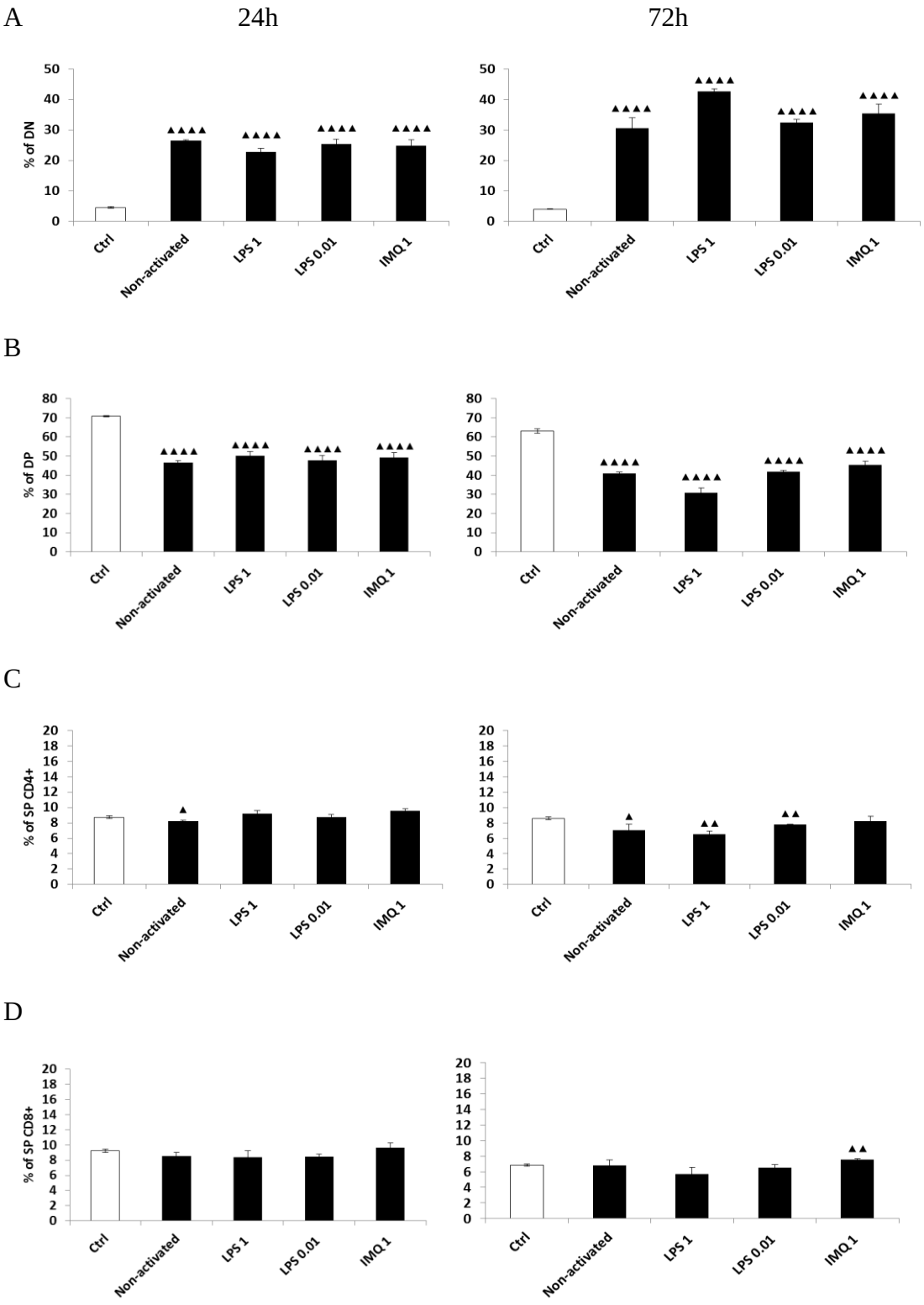


Figure 3.4:— Distribution of thymocyte subsets.
The experiments have been performed in triplicates. Thymocyte subsets distribution was analyzed after 24 and 72 h of *in vitro* culture in thymocytes culture alone (Ctrl, control non-pre-activated thymocytes) (white bars) and in co-culture with splenic B cells (black bars, thymocytes : B cells ratio 1:1). DN (A), DP (B), SP CD4+ (C), and SP CD8+ (D). The significance values were indicated as ▲ between Ctrl and co-cultures, and ● between non-activated and activated co-cultures. Ctrl (non-pre-activated thymocytes), non-activated (non-activated co-culture), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

Summary:

1. The presence of B cells in co-culture with thymocytes resulted in increased percentage of DN thymocytes and decreased percentage of DP thymocytes independently of B cell activation and duration of culture.
2. B cells did not change the distribution of SP CD4+ thymocytes in all conditions of 24 h culture, whereas influenced the percentage of these thymocytes in 72 h culture.
3. The presence of B cells in co-culture with thymocytes reduced the percentage of the SP CD8+ thymocytes in all conditions of culture independently on the duration of culture.

3.1.3. Viability of thymocytes in different culture conditions

The viability of DP, DN, SP CD4+, and SP CD8+ thymocytes in thymocytes culture alone (Ctrl) and co-cultures of thymocytes and B cells has been investigated in order to check if the changes of thymocyte subsets distribution resulted from differential survival of thymocytes or differences in their development. The results of our studies showed that the contact of thymocytes with B cells increased the viability of thymocytes or at least maintained the control level of viability (Fig. 3.5). The increase of the viability depended on the ratio of thymocytes to B cells, and was increased at 1:1 ratio. The viability of all thymocytes increased upon activation of B cells by the higher concentration of LPS, and this increase reached significance level in the ratio 1:1. In this setting, LPS1 and IMQ1 induced the increase of the viability compared to non-activated co-culture (DN), while only LPS1 increased the viability of SP CD8+ in co-culture compared to non-activated co-culture (Fig. 3.5 A and D).

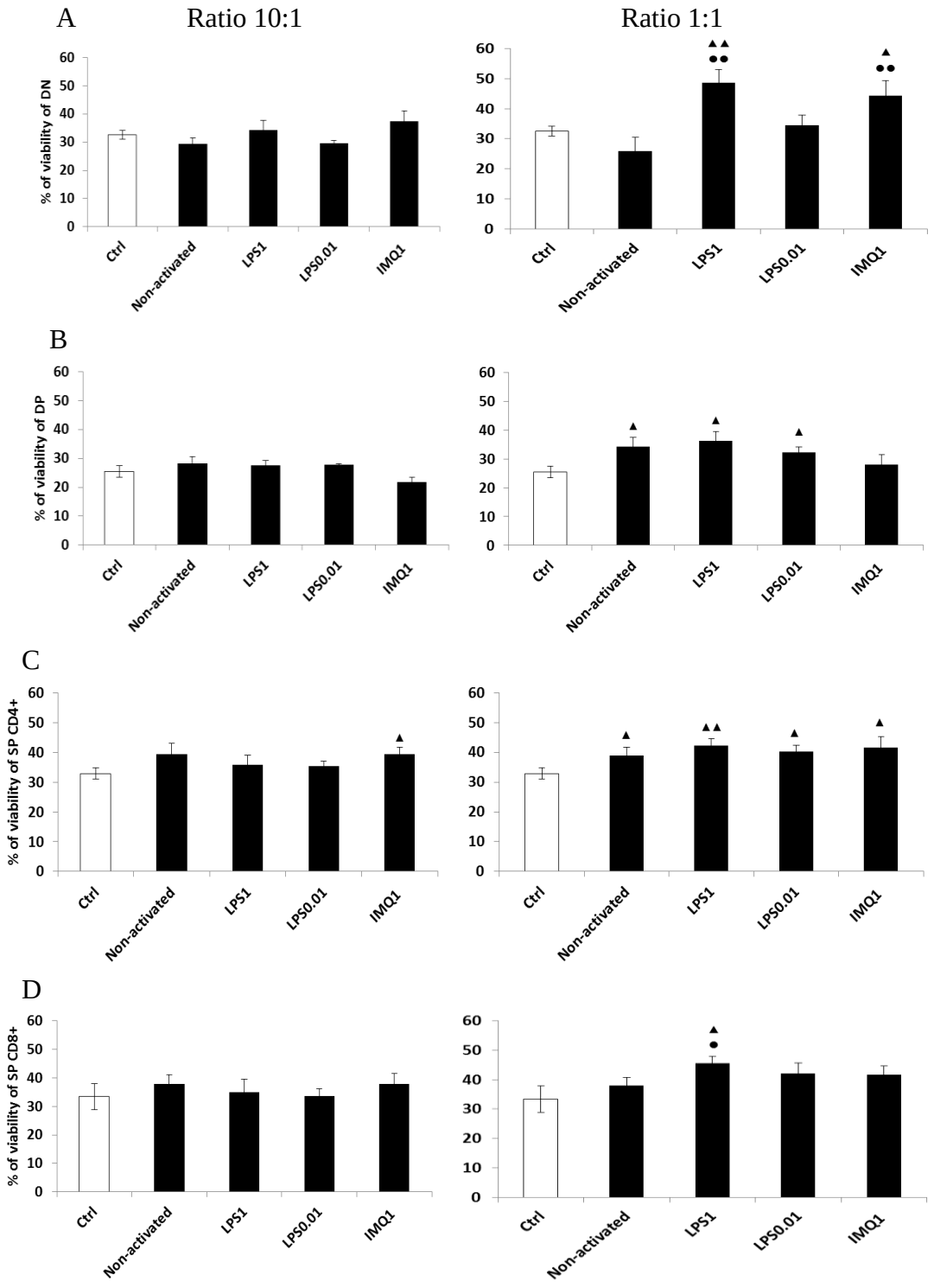


Figure 3.5:— Viability of thymocyte subsets.

DN (A), DP (B), SP CD4+ (C), and SP CD8+ (D) subsets in thymocytes culture alone as Ctrl (white bars) or in co-culture with splenic B cells (black bars) (thymocytes : B cells ratio 10:1 left), and (1:1 right) .

The experiments have been performed in triplicates. The cells were analyzed after 72 h of *in vitro* culture. The significance values were indicated as ▲ between Ctrl and co-cultures, and ● between non-activated and activated co-cultures. Ctrl (non-activated thymocytes), non-activated (non-activated co-culture), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

Summary:

1. The viability of all thymocytes (DN, DP and SP) co-cultured with B cells did not change substantially when B cells were not activated independently on thymocytes : B cells ratio (except cultures of 1:1 ratio, DP and SP CD4+).
2. The viability of DN thymocytes increased upon B cell activation and reached the highest percentage in LPS1- and IMQ1-conditioned media and depended on thymocytes : B cells ratio**.**
3. B cells to thymocytes ratio 1:1 was more efficient in increasing the viability of thymocytes. We suggest that the presence of B cells is crucial for thymocytes survival.
4. These results demonstrated that the distribution of thymocyte subsets in co-culture with B cells did not depend on differential survival potential of particular thymocyte subsets, but rather on the process of thymocyte development.

3.1.4. Generation of Foxp3+ thymus-derived Tregs in co-cultures of thymocytes and splenic B cells

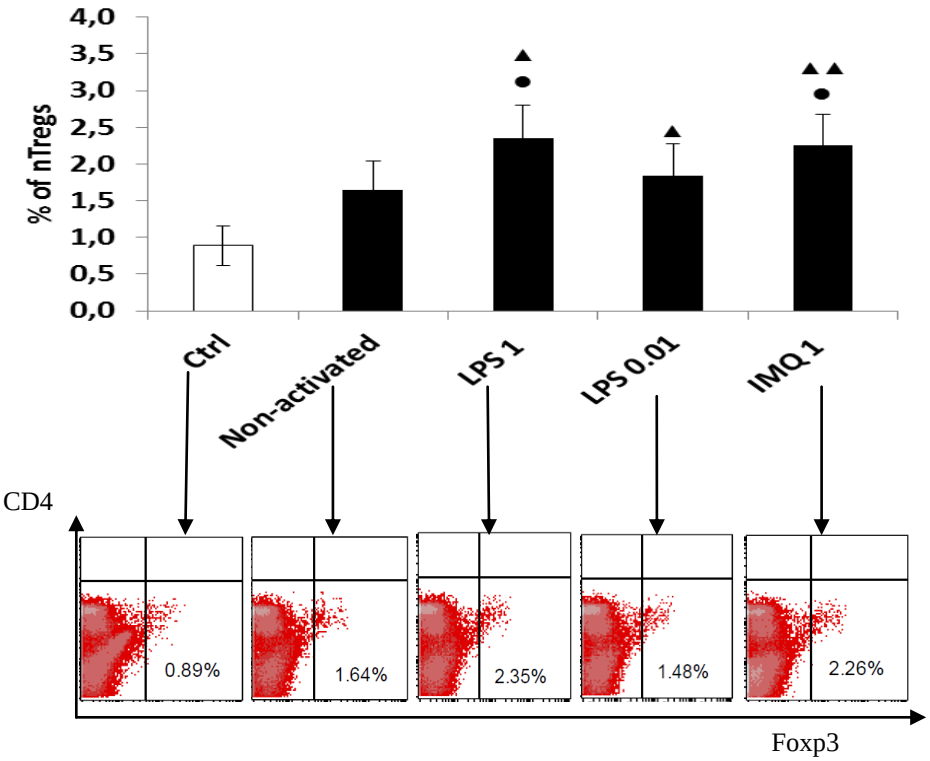
The percentage of mature nTregs in thymocytes co-cultured with splenic B cells has been studied. The percentage of terminally differentiated nTregs characterized by the expression of Foxp3 in SP CD4+ was analyzed (Fig. 3.6). The percentage of nTregs increased significantly in the presence of activated B cells in both 24 and 72 h co-cultures in both thymocytes : B cells ratios (Fig. 3.6). The increase of the percentage of nTregs was independent of the type of B cell activator and was similar for LPS and IMQ.

Short-term cultures (24 h) at 10:1 ratio resulted in the increase of nTregs only in the presence of activated B cells compared to incubation of thymocytes alone in culture medium (Fig. 3.6 A). When co-culture of thymocytes with splenic B cells extended to 72 h, the percentage of nTregs in all conditions of culture decreased compared to 24 h, however, the percentage of nTregs was still higher than in the culture of thymocytes alone both in the presence of activated and non-activated B cells (Fig. 3.6 B).

After changing of thymocytes : B cells ratio to 1:1 we observed statistically significant increase in the percentage of nTregs in the presence of non-activated and activated B cells independently on the type of TLR ligand used (Fig. 3.6 C and D). In 72 h cultures, the percentages of nTregs in all culture conditions were significantly

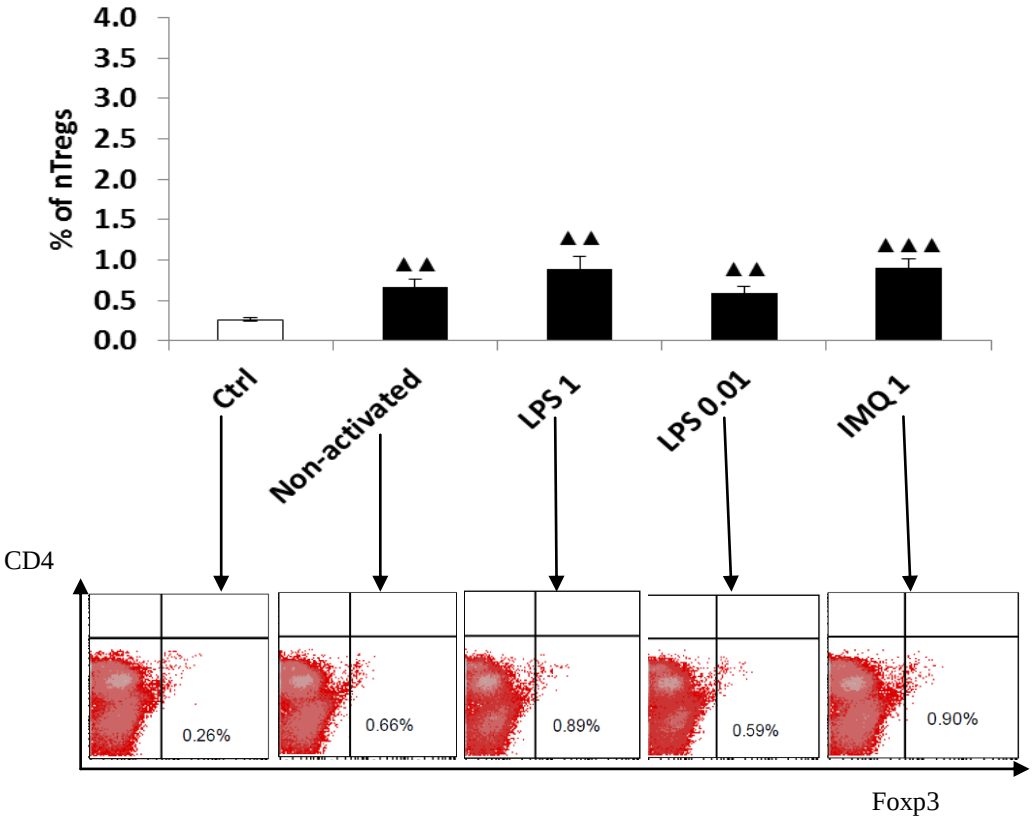
lower than in 24 h cultures. These results indicate that the availability of splenic B cells in co-cultures depends on their number, and thymocytes to B cells ratio might provide proportional signals important for thymic nTreg precursors to differentiate mature Foxp3 expressing nTregs. In addition, B cell activation provided a strong signal inducing nTreg generation. Accordingly, the results also suggested that role of the degree of B cells activation in relation to LPS concentration; however, the effect was not statistically significant.

A 24h



Thy : B=10:1

B 72h



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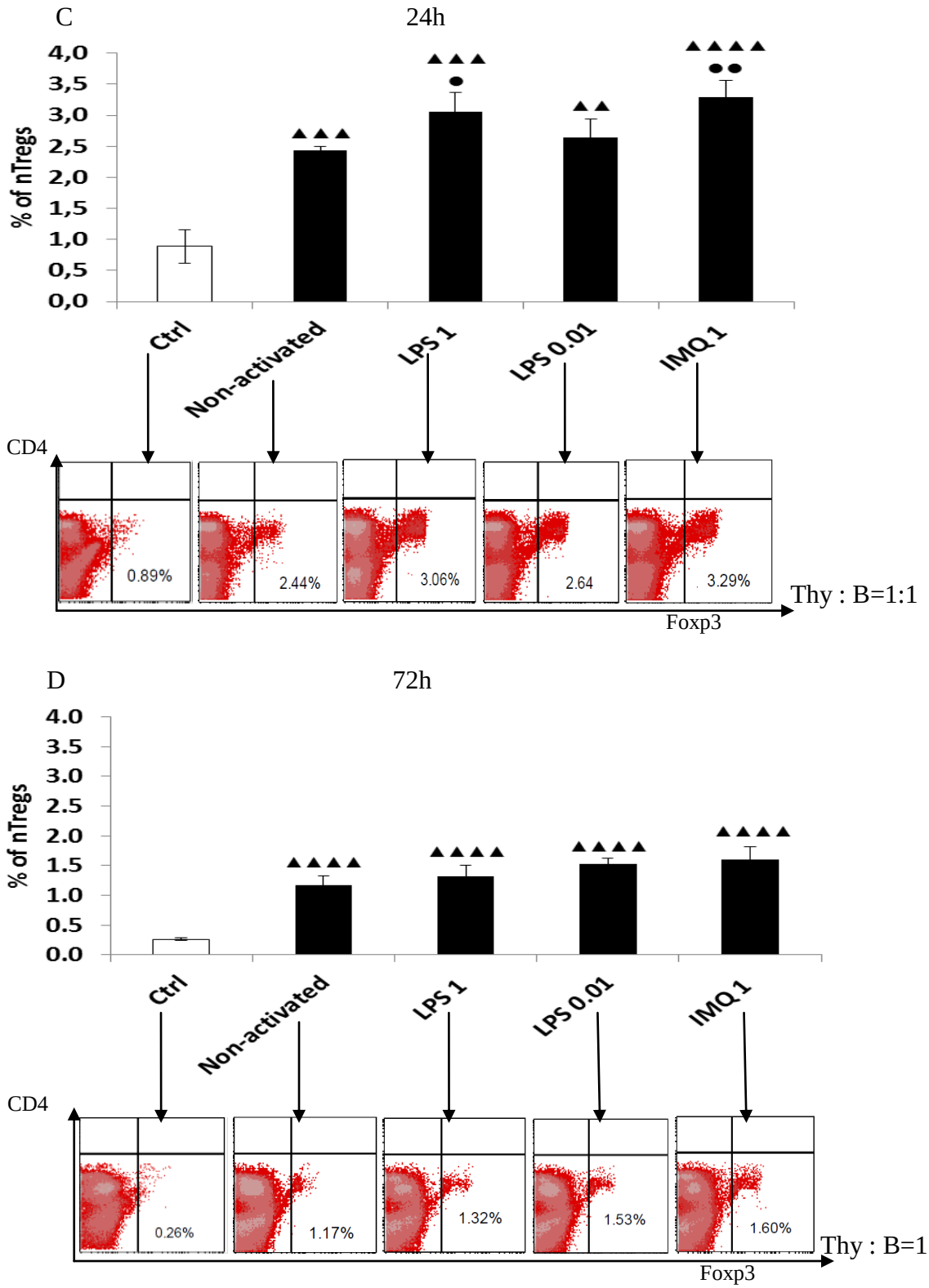


Figure 3.6:— Generation of CD4+Foxp3+ (nTreg) cells.
The experiments have been performed in triplicates. The cells were analyzed after 24 h (A and C) and 72 h (B and D) of *in vitro* culture in thymocytes culture alone as Ctrl. (white bars) or in co-culture with splenic B cells (black bars) (thymocytes : B cells ratio 10:1 A and B), and (ratio 1:1 C and D). The significance values were indicated as ▲ between Ctrl and co-cultures, and ● between non-activated and activated co-cultures. Ctrl (non-activated thymocytes), non-activated (non-activated co-culture), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

The prolongation of culture to 5 days demonstrated that additional signals should be provided to sustain the percentage of nTregs, however the pattern of changes in the co-cultures of thymocytes and activated B cells was maintained as in 24 h of culture (Fig. 3.7).

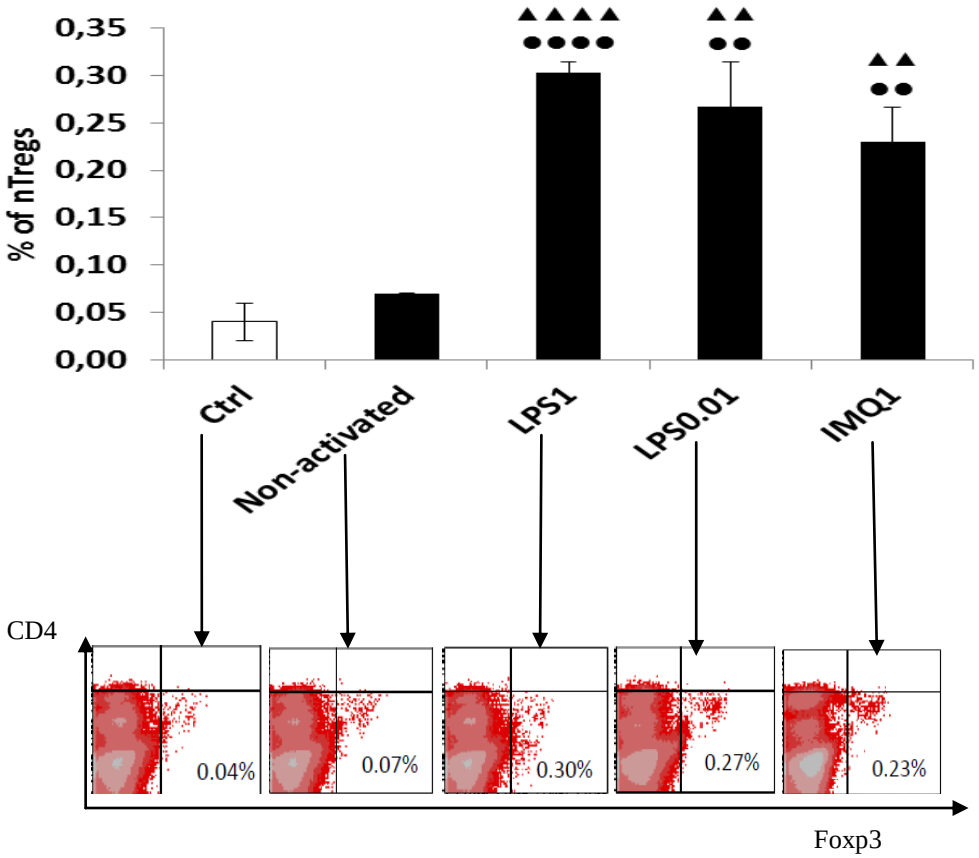


Figure 3.7:— Generation of CD4+Foxp3+ (nTreg) cells in long-term co-cultures with B cells.
The experiments have been performed in triplicates. The cells were analyzed after 5 days of *in vitro* culture in thymocytes culture alone as Ctrl (white bar) or in co-culture with splenic B cells (black bars) (thymocytes : B cells ratio 10:1). The significance values were indicated as ▲ between Ctrl and co-cultures, and ● between non-activated and activated co-cultures. Ctrl (non-activated thymocytes), non-activated (non-activated co-culture), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

Summary:

1. Splenic B cells increased the percentage of nTregs in co-cultures with thymocytes.
2. The increase of nTregs percentage depends on B cell activation, culture duration and thymocytes to B cells ratio.
3. The increase of the percentage of nTregs did not depend on the TLR ligands used in the study (LPS and IMQ).

3.1.5. Sequential steps of thymus-derived Treg development in co-cultures of thymocytes and splenic B cells

Sequential steps of nTreg development were analyzed as measured by the expression of CD25 and Foxp3 on SP CD4+ thymocytes. CD4+CD25+Foxp3– cells were considered immature nTregs and CD4+CD25+Foxp3+ cells fully mature nTregs. The percentage of immature CD4+CD25+ nTregs was increased in co-cultures with B cells, and the increase depended on the presence of B cells and their number rather than on their activation status (Fig. 3.8 A). The percentage of CD25 positive cells in SP CD4+ thymocytes in co-culture (independently on thymocytes to B cells ratio) increased in both non-activated and activated cultures compared to Ctrl. In addition, the increase was significant in all culture conditions, but was more significant in cultures of ratio 1:1 than in the other ratio (Fig. 3.8 A).

The percentage of mature CD4+CD25+Foxp3+ nTregs was strictly dose- and B cell ratio-dependent (Fig. 3.8 B). The percentage of nTregs in all culture conditions increased compared to Ctrl. Additionally, this percentage increased when B cells : thymocytes ratio increased from 1:10 to 1:1. When B cells were activated by LPS1 or IMQ1, the percentage was highly increased compared to non-activated and activated by LPS0.01 cultures (Fig. 3.8 B). Collectively, the analysis of sequential steps of nTregs development revealed that B cells have an indispensable role in the generation of nTregs and the percentage of these cells increased when B cells were activated through TLR4 and TLR7 ligands compared to non-activated cultures.

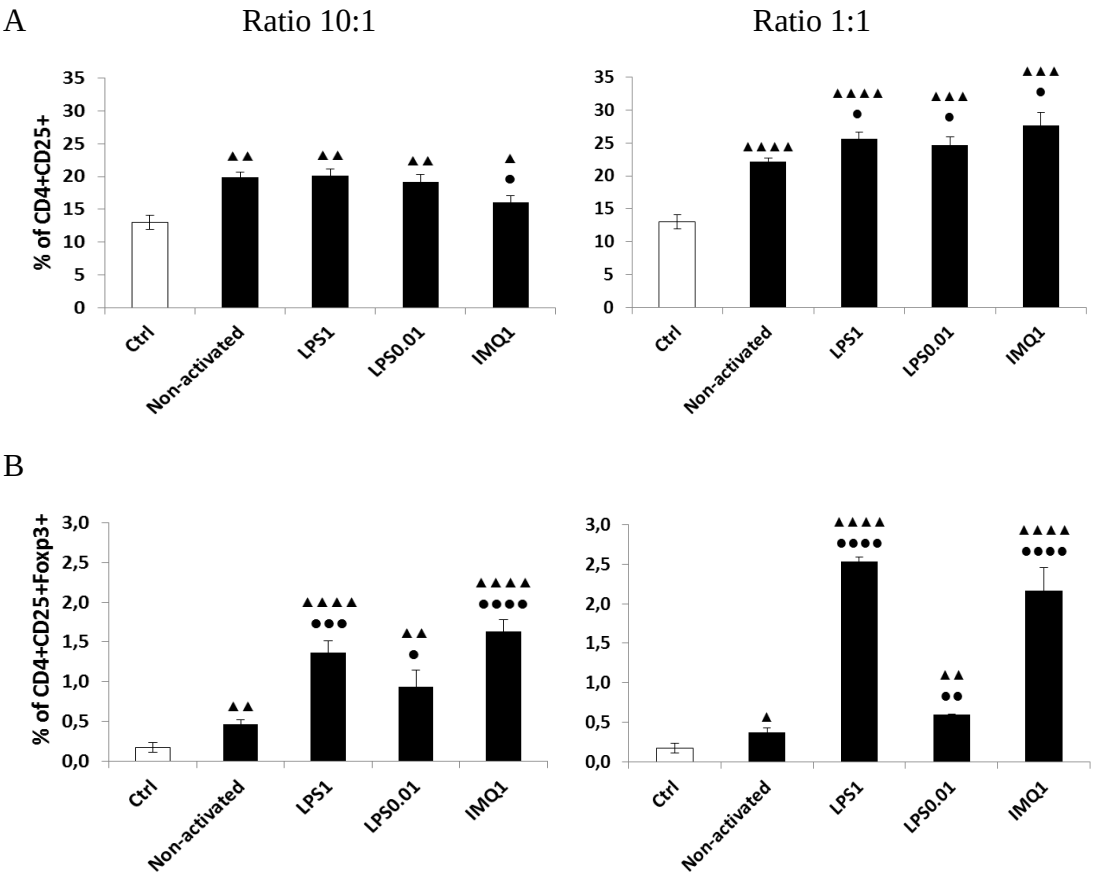


Figure 3.8:— Steps of nTreg maturation in co-cultures with B cells.
The percentage of CD25 positive thymocytes in SP CD4+ subset (A), and Foxp3 positive thymocytes in CD4+CD25+ subset (B).
The experiments have been performed in triplicates. The cells were analyzed after 72 h of *in vitro* culture in thymocytes culture alone as Ctrl (white bars) or in co-culture with isolated splenic B cells (black bars) (thymocytes : B cells ratio 10:1 left), and (ratio 1:1 right).
The significance values were indicated as ▲ between Ctrl and co-cultures, and ● between non-activated and activated co-cultures. Ctrl (non-activated thymocytes), non-activated (non-activated co-culture), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

Summary:

1. The co-culture of thymocytes and B cells resulted in the expression of CD25 on the SP CD4+ thymocytes and induction of Foxp3.
2. The expression of CD25 did not depend on the B cell activation but rather on thymocytes to B cells ratio.
3. The expression of Foxp3 strongly depends on the B cell activation, but not on the type of B cell activator.

3.1.6. Expression of Foxp3 in thymus-derived Tregs in different culture conditions

Considering the role of Foxp3 in the suppressive activity of nTregs we evaluated its expression in different culture conditions (Fig. 3.9). Our results revealed

clearly that the presence of B cells in culture of thymocytes (independently on thymocytes to B cells ratio) increased the expression of Foxp3 by developing nTregs. Additionally, our results revealed that the ratio of activated-splenic B cells has a crucial role in the expression of Foxp3 in nTregs (Fig. 3.9 A and B). The type of activator (LPS or IMQ) was of less importance.

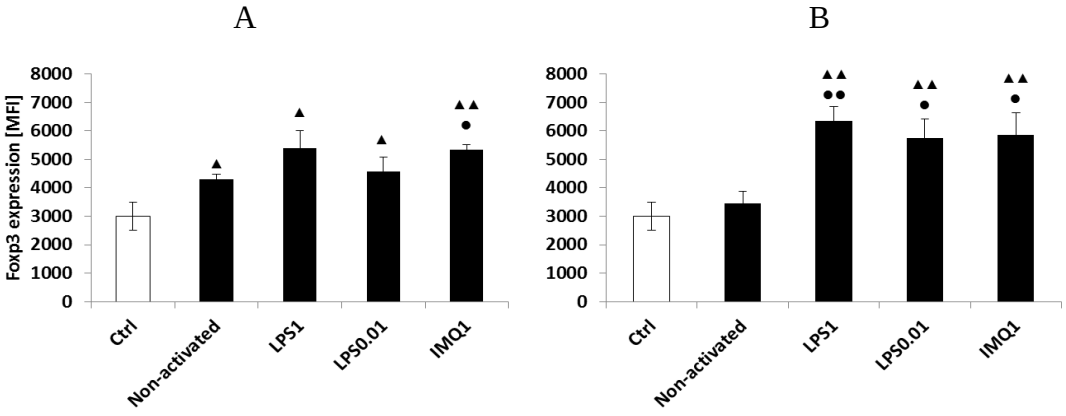


Figure 3.9:— The expression of Foxp3 molecule in nTreg cells in co-cultures with B cells.
The experiments have been performed in triplicates. The cells were analyzed after 72 h of *in vitro* culture in different conditions of thymocytes culture alone as Ctrl (white bars), or in co-culture with isolated splenic B cells (black bars) (thymocytes : B cells ratio 10:1 A), and (ratio 1:1 B).
The significance values were indicated as ▲ between Ctrl and co-cultures, and ● between non-activated and activated co-cultures. Ctrl (non-activated thymocytes), non-activated (non-activated co-culture), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

Summary:

1. The contact of thymocytes and splenic B cells resulted in the increase of the expression of Foxp3 independently on the ratio of thymocytes to B cells; however, the effect was strengthened by a higher number of B cells.
2. B cell activation increased the B cell-dependent up-regulation of Foxp3 in nTregs.
3. The type of activator was of minor importance in the increase of the expression of Foxp3.

3.1.7. Expression of co-stimulatory molecules and MHC II on splenic B cells in co-culture with thymocytes

To examine the role of contact between thymocytes and B cells on the antigen-presenting potential of B cells we studied the expression of co-stimulatory molecules and MHC II. The percentage of B cells positive for CD80 and CD86 increased in co-cultures with thymocytes after B cell activation; however, this percentage was lower than in the culture of splenic B cells alone (Fig. 3.10 A and B). We suggest that this may result from the presence of thymus-derived B cells

consuming LPS and IMQ either because of the expression of respective TLRs or changes of the kinetics of CD80 and CD86 expression caused by the contact between B cells and thymocytes. The percentage of CD40-positive B cells was lower in co-culture with thymocytes, however, in 72 h co-culture activation of B cells or presence of thymocytes showed the tendency to maintain the percentage of CD40-positive B cells diminished during the culture longer than 24 h (Fig. 3.10 C). The percentage of MHCII-positive B cells in co-cultures with thymocytes was decreased compared the culture of B cells alone reaching the control level in 72 h co-culture (Fig. 3.10 D).

In general, changes in the percentage of B cells expressing MHC II and co-stimulatory molecules were not caused by the contact with thymocytes, and the observed differences may reflect the activity of thymus-derived B cells taking part in the consumption of activators (TLR ligands). Indeed, our results revealed that the mere presence of thymocytes did not change the percentage of CD80 and CD86-positive B cells (except CD80/72 h). The percentage is increased by B cell activation, but at lower level than in cultures of B cells alone. We may suggest that thymic B cells compete with splenic B cells for TLR ligands, and the effects observed in B cell cultures alone are weakened in co-cultures with total thymic cells.

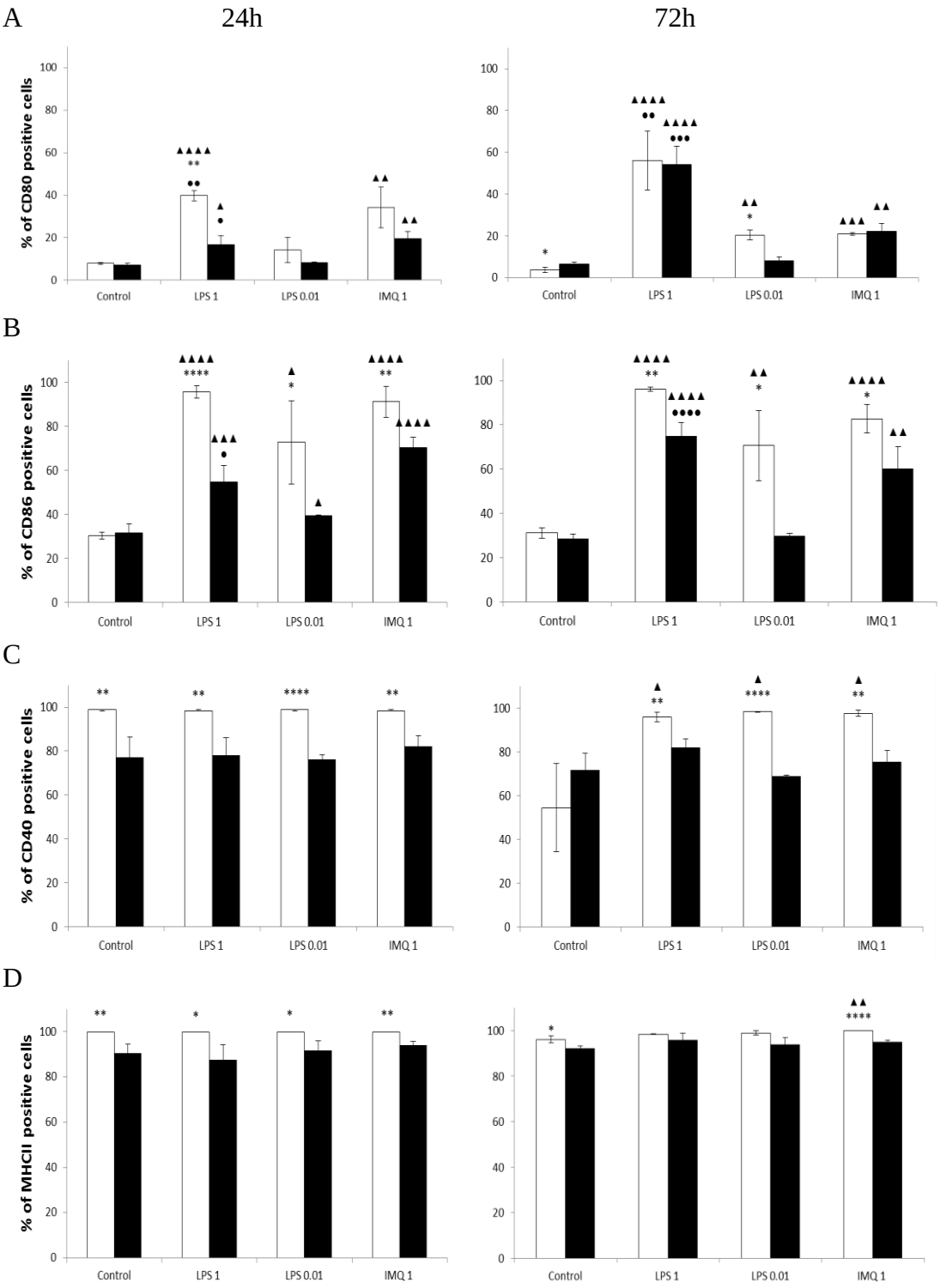


Figure 3.10:— The percentage of co-stimulatory molecules and MHC II expressing B cells in co-cultures with thymocytes.

CD80 (A), CD86 (B), CD40 (C), and MHC II (D). White bars represent B cells cultured alone; black bars represent B cells co-cultured with thymocytes in thymocytes : B cells ratio 10:1. The experiments have been performed in triplicates. The cells were analyzed after 24 and 72 h of *in vitro* culture. The significance values were indicated as▲ between Control (non-activated) and activated B cells in 24 or 72 h cultures, ● between the concentrations of LPS or IMQ for the same time of culture, and * between 24 and 72 h cultures. Control (non-activated cells), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), IMQ5 (5µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for other symbols).

We have observed that the expression of CD80 on B cells after 24 and 72 h co-cultures increased compared to splenocytes cultured alone and the increase was statistically significant upon activation by LPS1 (Fig. 3.11 A). The mere presence of thymocytes in co-cultures resulted in up-regulation of CD86 in both durations of culture. After activation, the expression of this molecule decreased in 24 h co-cultures compared to splenocytes cultured alone (Fig. 3.11 B). The expression of CD40 and MHC II was also decreased in co-culture with thymocytes (Fig. 3.11 C and D). The results of this part of the study revealed that the presence of thymocytes in activated co-culture induced the up-regulation of CD80 and down-regulation of CD86, CD40, and MHC II and these changes were time and type of activator-dependent.

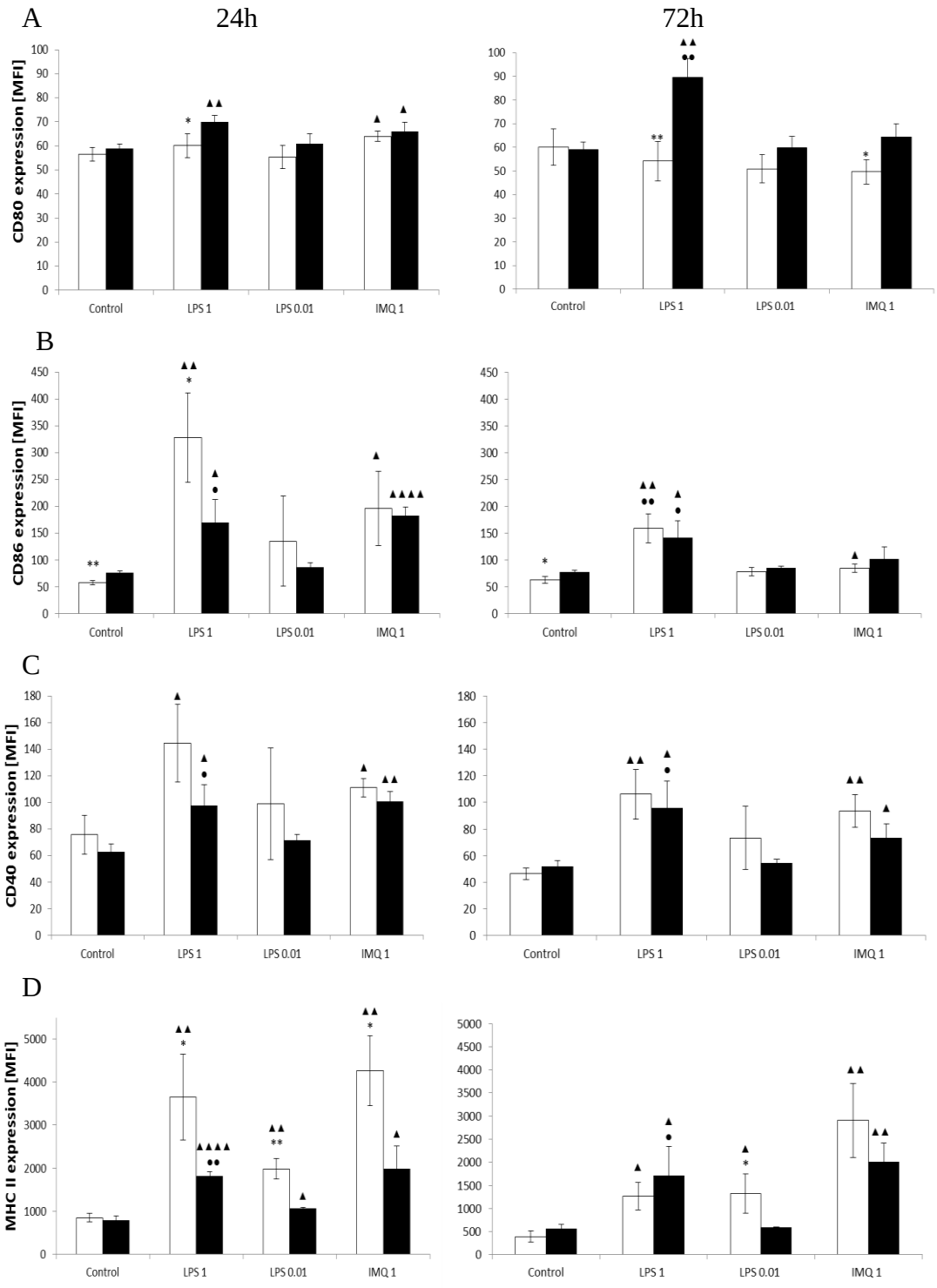


Figure 3.11:— The expression of co-stimulatory molecules and MHC II on B cells co-cultured with thymocytes.

CD80 (A); CD86 (B); CD40 (C) and MHC II (D). White bars represent B cells cultured alone; black bars represent B cells co-cultured with thymocytes in thymocytes : B cells ratio 10:1. The experiments have been performed in triplicates. The cells were analyzed after 24 and 72 h of *in vitro* culture. The significance values were indicated as▲ between Control (non-activated) and activated B cells in 24 or 72 h cultures, ● between the concentrations of LPS or IMQ for the same time of culture, and * between 24 and 72 h cultures. Control (non-activated cells), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), IMQ5 (5µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for other symbols).

Summary:

1. The mere presence of B cells did not affect the percentage of CD80 and CD86 positive splenic B cells, and these percentages were increased upon activation by LPS1 and IMQ1 even if the percentage was lower than in splenocytes culture alone under the same culture conditions.
2. The level of CD80 in co-culture was higher than of the same condition for splenocytes culture alone.

3.1.8. Role of co-stimulatory molecules and MHC II blocking on thymus-derived Treg generation in co-culture of thymocytes and splenic B cells

To determine the role of co-stimulatory molecules in nTreg generation blockade of these molecules on B cells was performed. This part of the study consisted of the following steps:1/ multiple molecule blockade using anti-CD80 + anti-CD86 + anti-CD40 + anti-MHC II simultaneously; 2/ single molecule blockade using anti-CD80, anti-CD86, anti-CD40, and anti-MHC II separately; 3/ paired blockade using anti-MHC II and anti-co-stimulatory molecule, namely: anti-CD80 + anti-MHC II, anti-CD86 + anti-MHC II, and anti-CD40 + anti-MHC II. The results of blocking experiments are presented on Fig. 3.12.

We showed that the blockade of all analyzed molecules resulted in more than 5-fold decrease in percentage of nTregs compared to activated sample (Fig. 3.12 a). The effect of both B7 molecules in the generation of nTregs was evenly matched between each other by decreasing the percentage of nTregs generation 2-fold compared with activated samples but the effect of CD86 was stronger than the effect of CD80 and for both strategies of blockade (anti-B7 or anti-B7 + anti-MHC II) (Fig. 3.12 b, c, f, and g). CD40 seemed to be less important as it decreased the percentage of nTregs 1-fold only (Fig. 3-12 d). However, this inhibitory effect was more significant in combination with MHC II (Fig. 3.12 h).

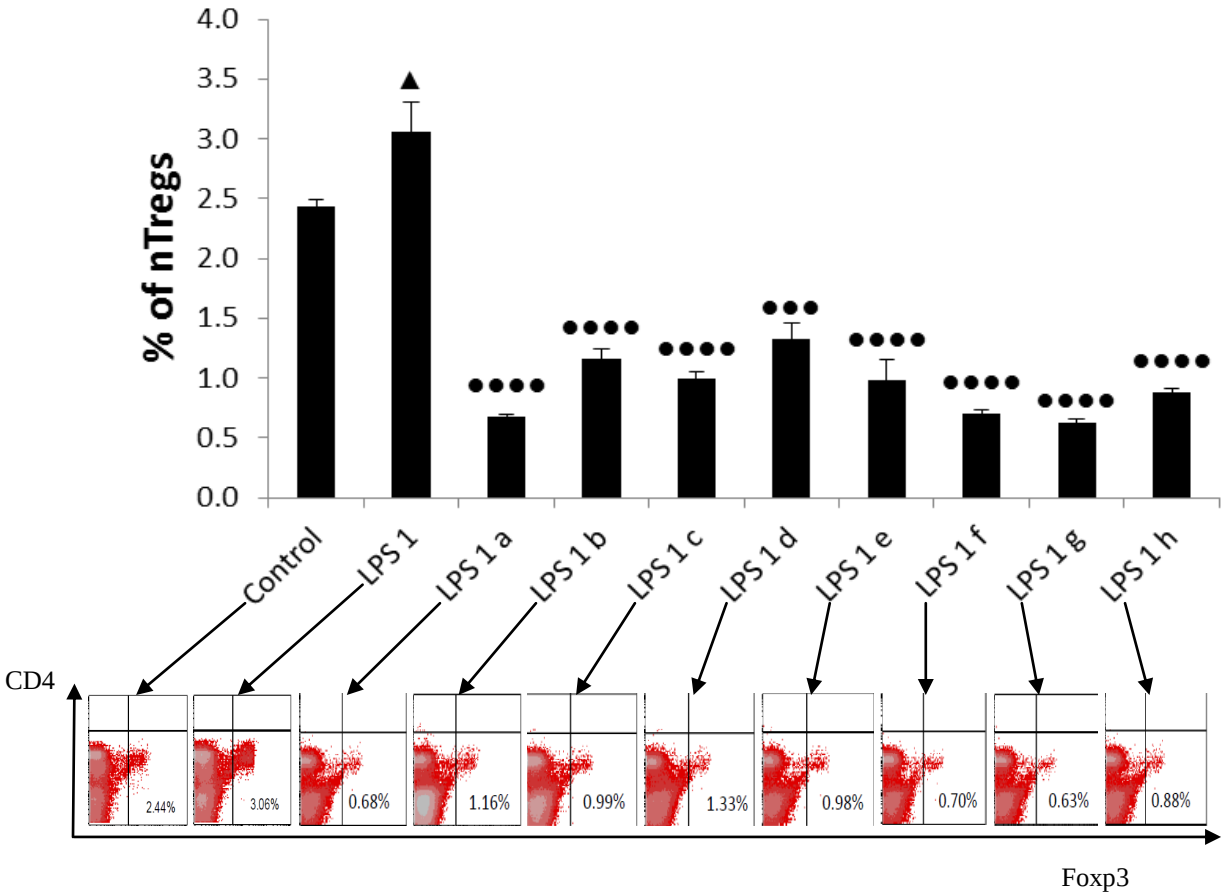


Figure 3.12:— Effect of MHC II and co-stimulatory molecules blockade on nTreg generation.
The cultures were activated by LPS1 (1µg/ml). The cells were analyzed after 24 h of *in vitro* co-culture with thymocytes (thymocytes : B cells ratio 1:1). The significance values were indicated as▲ between Control (non-activated cells) and activated culture, and ● between activated culture and blockade cultures. The small letters beside activator indicated as following: CD80+CD86+CD40+MHC II molecules have been blocked (a), CD80 molecule has been blocked (b), CD86 molecule has been blocked (c), CD40 molecule has been blocked (d), MHC II molecule has been blocked (e), CD80+MHC II molecules have been blocked (f), CD86+MHC II molecules have been blocked (g), and CD40+MHC II molecules have been blocked (h). The levels of probability were indicated as follows (●$p < 0.05$; ●●$p < 0.01$; ●●●$p < 0.001$; and ●●●●$p < 0.0001$, and similarly for ▲).

Summary:

1. All molecules (MHC II, CD80, CD86, and CD40) play a role in the generation of nTregs.
2. Blocking of pairs of molecules (MHC II and one co-stimulatory) and blocking all of them resulted in the strong inhibition of nTreg generation. We suggest that CD86 highly influence nTreg generation in our experimental model, and the decrease of nTregs percentage is as strong as in the case of blockade of all the studied molecules.

3.1.9. Biological activity of thymus-derived Tregs generated in co-cultures with splenic B cells

We have investigated the biological activity of *in vitro* generated CD4+Foxp3+ nTregs using the functional assay based on the suppressive activity of nTregs on the proliferation of responder activated CD4+ T cells. Anti-CD3 (0.5 µg/ml) and anti-CD28 (0.05 µg/ml) were used for CD4+ T cells activation thereby to induce proliferation of these cells. (Fig. 3.13).

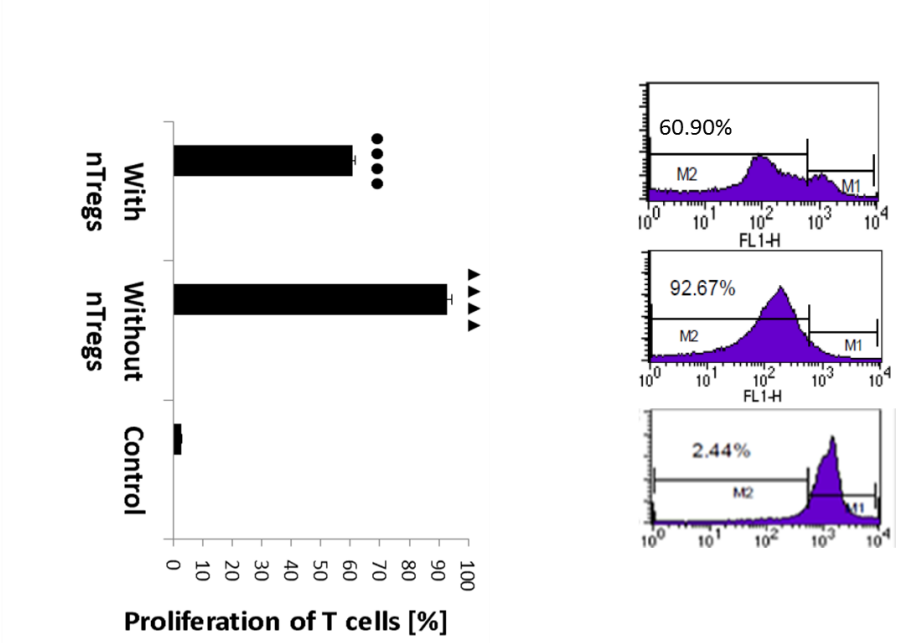


Figure 3.13:— Biological activity of *in vitro* generated nTregs.
Non-activated responder T cells (Control), activated responder T cells (Without nTregs), and co-culture of activated responder T cells & nTregs isolates from co-culture with B cells activated with LPS (With nTregs).
The experiments have been performed in triplicates. The cells were analyzed after 72 h of *in vitro* culture. The significance values were indicated as▲ between (without nTregs) and (control), and ● between (without nTregs) and (with nTregs). The percentage of inhibition was 34.28% in (with nTregs culture), according to equation described in materials and methods section. The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

The results demonstrated that nTregs in the co-culture of thymocytes with LPS-activated splenic B cells showed the suppressive activity against activated target CD4+ T cells.

Summary:

1. Thymus-derived Tregs generated in the co-culture of thymocytes and activated B cells exhibited the potential to inhibit the proliferation of activated responder T cells.

3.1.10. Thymocyte subsets distribution in co-cultures of anti-CD3 pre-activated thymocytes and splenic B cells

We have demonstrated that in the population of nTreg precursors exposed to weak signal from TCR and strong signal from co-stimulatory molecule receptors the percentage of Foxp3+ thymocytes in CD4+ subset was increased in 24 h co-cultures. The percentage of nTregs was still higher in 72 h co-cultures; however, the absolute value was lower than in 24 h co-cultures (Fig. 3.6). To demonstrate the effect of strong signal from TCR on nTreg generation in co-cultures anti-CD3 pre-activated thymocytes with B cells activated longer than 24 h have been performed. In this modified conditions of co-culture the previous experimental variant was performed. The 72 h of culture was chosen to demonstrate the effect of the strong signal.

The effect of anti-CD3 pre-activation of thymocytes on the distribution of thymocyte subsets in the co-cultures with LPS- or IMQ-activated B cells has been investigated.

Thymocyte pre-activation by CD3 monoclonal antibodies and further co-culture with B cells resulted in strong increase of the percentage of DN and decrease of the percentage of DP thymocytes, which can suggest a developmental block at the DN to DP stage or DP differentiation into SP thymocyte subsets. At the same time the increase of the percentage of SP CD4+ was observed in the presence of B cells activated by the higher concentration of LPS. The increase of the percentage of SP CD8+ thymocytes was observed in all co-culture conditions.

LPS1-activated B cells decreased the percentage of DN thymocytes, whereas activation by LPS0.01 and IMQ1 decreased this subset compared to the co-culture containing non-activated B cells (Fig. 3.14 A). LPS1-activated B cells decreased the percentage of DP thymocytes, and activation by LPS0.01 and IMQ1 increased this subset compared to the co-culture containing non-activated B cells (Fig. 3.14 B). Interestingly, LPS1-activated B cells significantly increased the percentage of SP CD4+ thymocytes whereas the activation by low dose of LPS decreased the percentage of this subset compared to the co-culture containing non-activated B cells (Fig. 3.14 C). Both low doses of activators significantly increased the percentage of SP CD8+ thymocytes whereas higher dose of LPS did not affect the percentage of this subset compared to the co-culture containing non-activated B cells (Fig. 3.14 D).

Lack of anti-CD3 pre-activation step, as we demonstrated in previous section (3.1.2), resulted in decreased percentage of both SP CD4+ and SP CD8+ thymocytes.

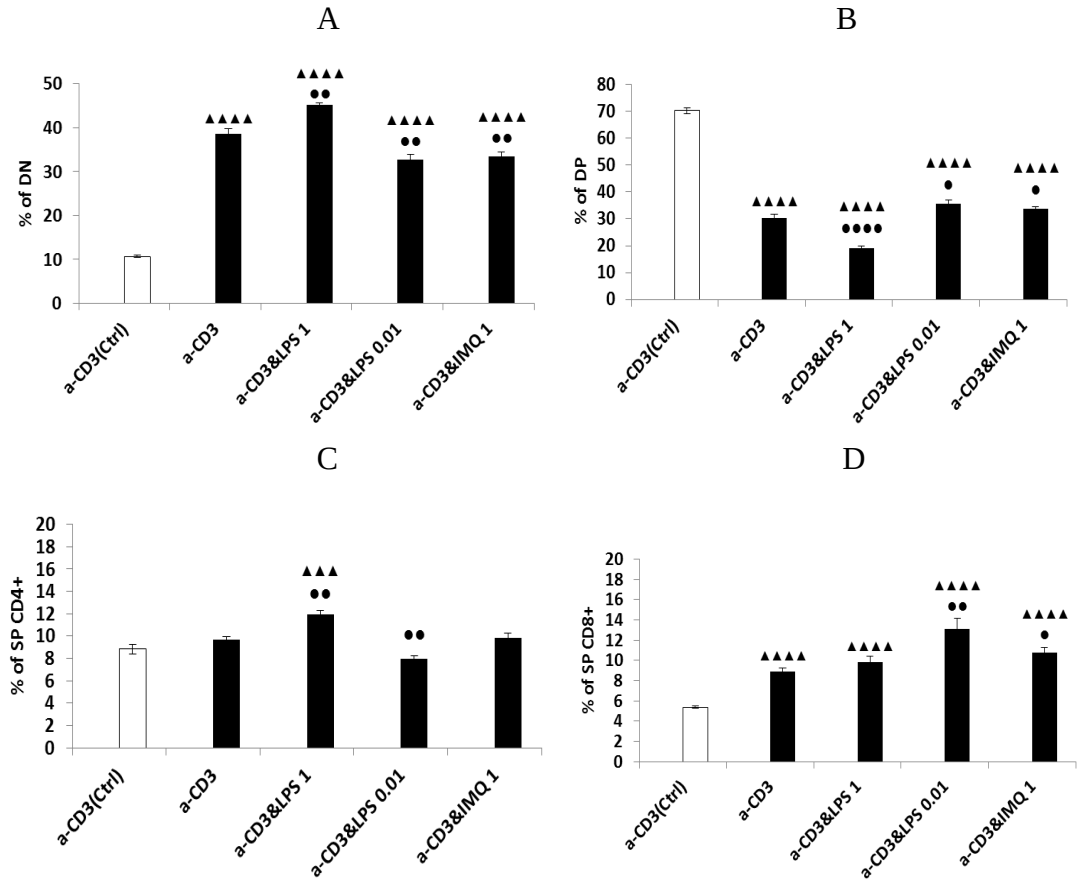


Figure 3.14:— Effect of anti-CD3 thymocyte pre-activation on the distribution of the main thymocyte subsets.

A/ DN thymocytes; B/ DP thymocytes; C/ SP CD4+ thymocytes; D/ SP CD8+ thymocytes. Ctrl, a-CD3 (white bars): thymocytes cultured alone without B cells; black bars: a-CD3 pre-activated thymocytes co-cultured with non-activated B cells and activated by LPS or IMQ (thymocytes : B cells ratio 1:1).

The experiments have been performed in triplicates. The cells were analyzed after 72 h of *in vitro* cultures. The significance values were indicated as ▲ between ctrl and co-cultures, and ● between non-activated and activated B cells. anti-CD3(Ctrl) (pre-activated thymocytes), a-CD3 (pre-activated co-culture), a-CD3&LPS1 (pre-activated+LPS1µg/ml), a-CD3&LPS0.01(pre-activated+LPS 0.01µg/ ml), and a-CD3& IMQ1(pre-activated+IMQ 1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

Summary:

1. Anti-CD3 pre-activation of thymocytes followed by co-culture with B cells led to the increase of SP thymocyte subsets, mainly SP CD8+. The activation of B cells in this process was of minor importance for SP CD8+ differentiation.
2. The increase of the percentage of SP CD4+ thymocytes was statistically significant only after B cell activation by high dose LPS.

3. The presence of B cells resulted in the strong increase of DN and decrease of DP thymocytes.

3.1.11. Generation of Foxp3+ thymus-derived Tregs in co-cultures of anti-CD3 pre-activated thymocytes and splenic B cells

The content of nTregs was evaluated in 72 h co-cultures of CD3-pre-activated thymocytes and B cells (Fig. 3.15). Pre-activation of thymocytes by CD3 monoclonal antibodies and further co-culture with B cells resulted in the strong increase of nTregs. We found that the presence of splenic B cells was important when thymocytes received strong signal via TCR by anti-CD3 activation, as it caused the increased generation of nTregs. In these conditions (anti-CD3 activation) the activation of B cells was of minor importance.

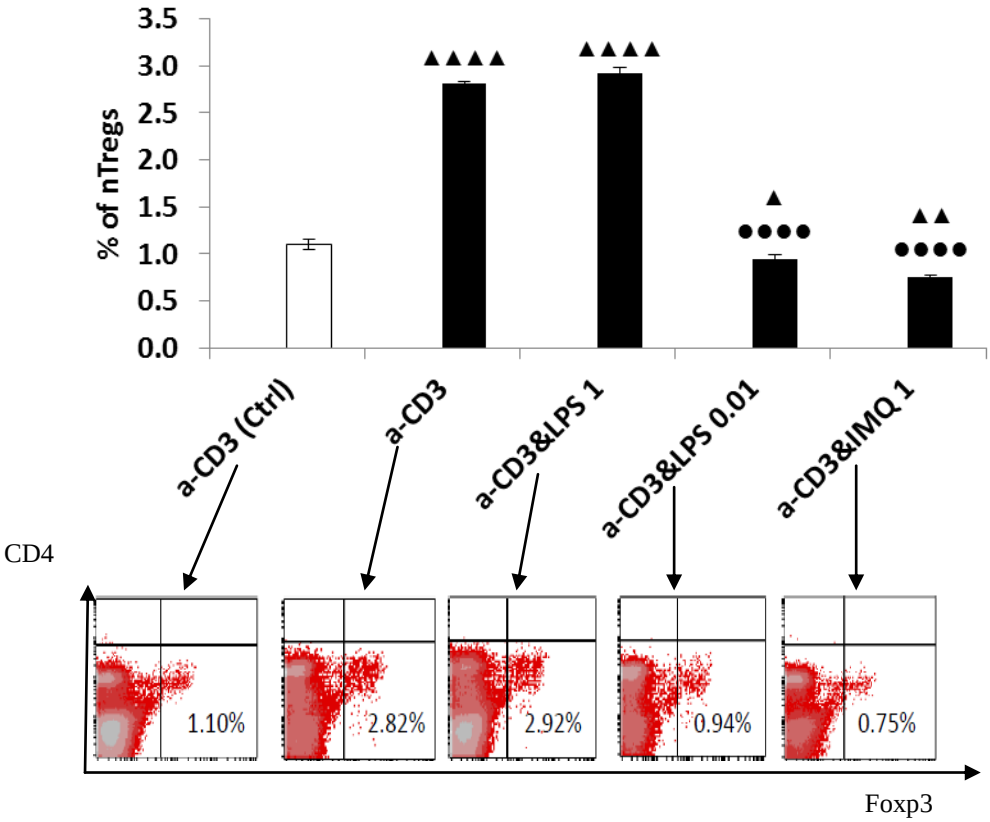


Figure 3.15:— Percentage of CD4+Foxp3+ (nTregs) in co-cultures of anti-CD3 pre-activated thymocytes and B cells.

Ctrl, anti-CD3 (white bar): thymocytes cultured alone without B cells; black bars: anti-CD3 pre-activated thymocytes co-cultured with non-activated B cells and activated by LPS or IMQ (thymocytes : B cells ratio 1:1). The experiments have been performed in triplicates. The cells were analyzed after 72 h of *in vitro* cultures. The significance values were indicated as ▲ between ctrl and co-cultures, and ● between non-activated and activated B cells. anti-CD3(Ctrl) (pre-activated thymocytes), anti-CD3 (pre-activated co-culture), a-CD3&LPS1 (pre-activated+LPS1µg/ml), a-CD3&LPS0.01(pre-activated+LPS 0.01µg/ml), and a-CD3& IMQ1(pre-activated+IMQ 1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

LPS1-activated B cells increased the percentage of nTregs, whereas B cells activated by either LPS0.01 or IMQ1 significantly decreased the percentage of nTregs compared to either pre-activated thymocytes or non-activated B cells (Fig. 3.15). This result strongly suggested the role of B cells in the generation of nTregs because the presence of non-activated B cells increased the number of nTregs. After B cell activation, when B cells received strong signal from TLR4 ligand (LPS1 activation), the percentage of generated nTreg was maintained while when B cells received weak signal from either TLR4 or TLR7 (activation by LPS0.01 or IMQ1) the percentage of generated nTreg significantly decreased compared to pre-activated thymocytes (Ctrl). We cannot give any scientific interpretation to the observed decrease in number of

nTreg after pre-activation and activation by low doses of both activators. To fully understand these observations further studies are required.

Summary:

1. Pre-activation of thymocytes by CD3 monoclonal antibodies followed by co-culture with B cells resulted in increase of nTregs. The activation of B cells was of minor importance after administration of high dose LPS.
2. Low concentration of LPS and IMQ counteracted the effect of anti-CD3 pre-activation.

3.1.12. The role of co-stimulatory molecules and MHC II blocking on thymus-derived Treg generation in co-culture of anti-CD3 pre-activated thymocytes and splenic B cells

To determine the role of co-stimulatory molecules and MHC II expressed on splenic B cells in nTreg generation blocking antibodies were used. In this step the co-cultures were incubated for 24 h only as because the after this time the highest percentage of generated nTregs was observed.

Our results have revealed that blockade of all studied molecules decreased the percentage of nTregs (Fig. 3.16). The strongest inhibition of generation of nTreg was observed in the after CD86 and CD80 blockade; however, the effects of CD40 blockade were also evident. Simultaneous blockade of all molecules led to absolute abolition of nTreg generation. These results are in accordance with previously presented data (Fig. 3.12) showing the results of molecules blockade in thymocytes co-cultures with activated B cells.

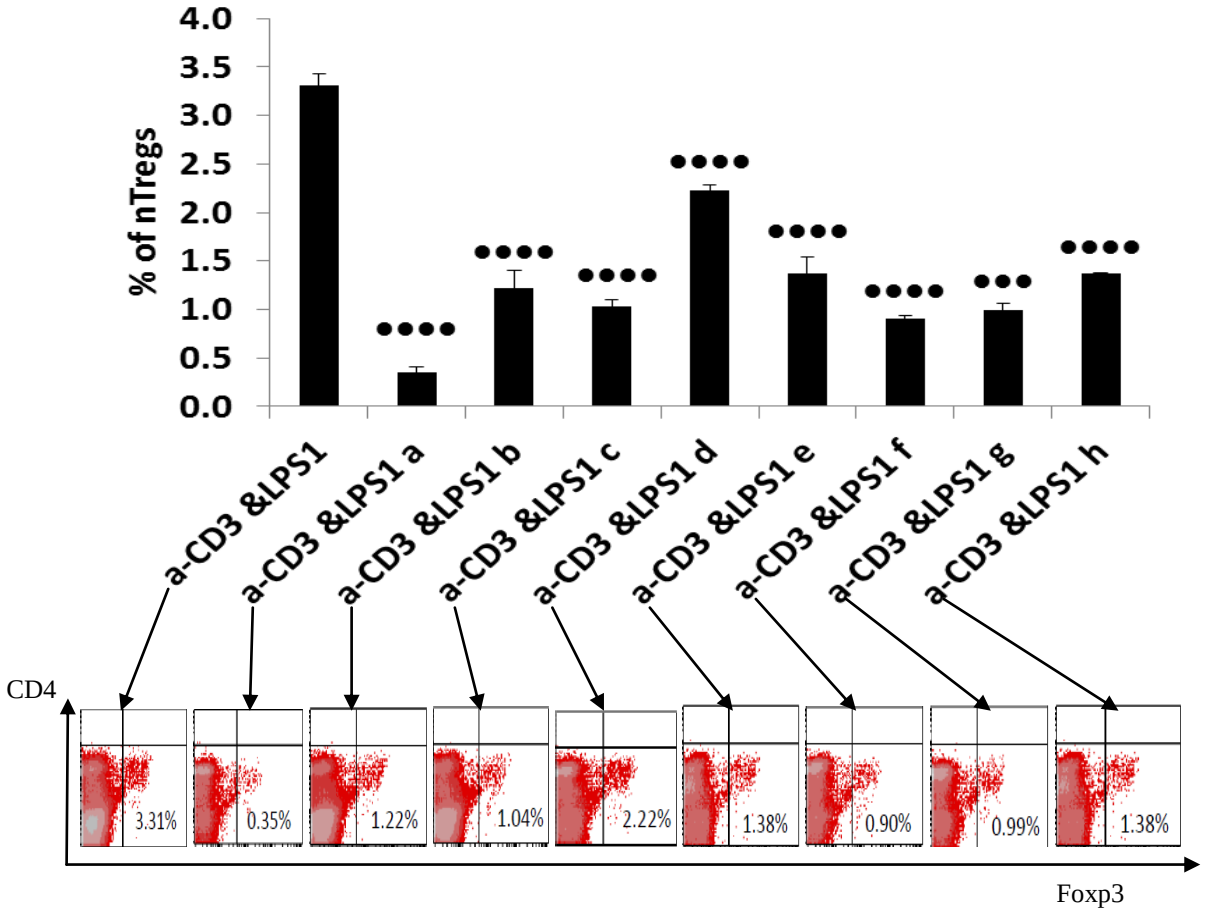


Figure 3.16:— The effect of co-stimulatory molecules and MHC II blockade on nTreg generation. The cells were analyzed after 24 h of *in vitro* co-culture with thymocytes (thymocytes : B cells ratio 1:1). The significance values were indicated as ● between blockade samples and a-CD3&LPS1 sample. The small letters beside activator indicated as following: CD80+CD86+CD40+MHC II molecules have been blocked (a), CD80 molecule has been blocked (b), CD86 molecule has been blocked (c), CD40 molecule has been blocked (d), MHC II molecule has been blocked (e), CD80+MHC II molecules have been blocked (f), CD86+MHC II molecules have been blocked (g), and CD40+MHC II molecules have been blocked (h). The levels of probability were indicated as follows (●$p < 0.05$; ●●$p < 0.01$; ●●●$p < 0.001$; and ●●●●$p < 0.0001$).

Summary:

1. MHC II, CD80, CD86, and CD40 play an important role in the generation of nTregs.
2. The results of our studies showed that CD80/CD86 molecules are of major importance for nTreg development.

Discussion

We hypothesized that B cells can be involved in the generation of natural regulatory T cells. Our hypothesis was built on the following facts: firstly, the normal development of nTreg in the thymus requires interaction with thymic antigen-presenting cells such as dendritic cells and thymic epithelial cells (van Santen et al., 2004; Pennington et al., 2006). These cells present antigens to developing nTreg for

their selection and maturation (Jenkinson et al., 1994; Volkmann et al., 1997). Secondly, it was found that a minor percentage of B cells reside in the thymus, and the precise function and origin of these cells are still controversial (Kleindienst et al., 2000; Hashimoto et al., 2002; Ceredig et al., 2007). In addition, nTreg cells have a crucial role in maintaining the immune system homeostasis and control the pathological and physiological responses. The percentage of these cells is limited, and they comprise about 4% of SP CD4+ cells (Fontenot et al., 2005). We introduced a model of co-culture of thymocytes with splenic B cells to investigate if B cells, which can serve as antigen-presenting cells for peptide antigens (Vascotto et al., 2007; Taneja et al., 2007; Roux and Niedergang, 2012), have the potential to induce the generation of nTregs in *in vitro*.

Recently it was documented that many phenotypically different B cells can be involved in the induction of Tregs in peripheral lymphoid organs (Chen et al., 2009; Zheng et al., 2010). In addition, it was shown that B cells are available in the thymus and are involved in negative selection of autoreactive CD4+ thymocytes (Frommer and Waisman, 2010). Our model was created to examine if peripheral B cells can influence the generation of nTregs from thymic precursor cells. Based on the results of Bieńkowska et al. (Bieńkowska et al., 2014) on the role of different sources of activatory signals necessary to the generation of thymic Tregs, we have used splenic B cells as antigen-presenting cells providing necessary signals for nTreg maturation.

It is known that B cells can serve as antigen-presenting cells and we have confirmed it in our data by showing the expression of co-stimulatory and MHC II molecules on splenic B cells activated through TLR4 and TLR7 (bacterial and viral infection-mimicking agents which represent the common pathogens for our body). In addition, these molecules are important for further interaction with conventional T cells during their activation (Chambers, 2001; Salomon and Bluestone, 2001). Accumulated evidence indicates that CD28/B7 interactions not only regulate T cell activation but are also required for the development (Tang et al., 2003; Liang et al., 2005) and homeostasis of CD4+CD25+ Tregs (Salomon et al., 2000).The expression of CD80, CD86, and CD40 have been investigated because these molecules are crucial in the signal transduction to T cells playing a role of co-stimulatory molecules for T cells activation and differentiation into effector populations (van Kooten and Banchereau, 1997; Tang et al., 2003; Liang et al., 2005; Lu et al., 2015). Splenic B cells constitute a large population of lymphocytes with full capacity to internalize, process and

present antigen to T cells. For instance, *in vitro* studies have found that the ability of activated B cells to activate naïve CD4+ T cells is almost as high as that of DCs (Cassell and Schwartz, 1994). On the other hand, *in vitro* responses of B cells to LPS or IMQ are different, and the outcome of such stimulation is strongly dose-dependent (Coutinho et al., 1974; Dziarski, 1982; Suvas et al., 2002; Sauder, 2003). In this regard, LPS can modulate antigen internalization and regulate different molecules in dose dependent manner (Xu et al., 2008). Our results revealed that a significant population of splenic B cells expresses co-stimulatory molecules and MHC II and their expression were up-regulated after 24 h upon activation by T-independent stimuli. This result is consistent with the results of other researchers who have observed up-regulation of co-stimulatory molecules upon activation (Suvas et al., 2002; Xu et al., 2008). In our culture conditions B cells constitutively express low level of CD80. This result differs from the results of others which revealed that B cells did not normally express CD80 but this molecule could be inducible (Lenschow et al., 1996).

We examined the effect of the presence of B cells on the distribution of the main thymocyte subsets and concluded that B cells increase SP CD4+ content as was observed in co-cultures of thymocytes and JAWS II cells acting as antigen-presenting cells (Bieńkowska, 2014). These experiments were performed to see if the contact of thymocytes with B cells (non-activated or activated through TLR4 or TLR7) results in the differentiation toward SP CD4+ thymocytes which can further mature to nTregs. The increase of DN thymocytes and the decrease of DP thymocytes (in LPS and IMQ-activated and anti-CD3-pre-activated co-cultures) may result from the developmental block at the DN/DP stage, differentiation of DP to SP or differential sensitivity of thymocyte subsets to apoptosis manifested by changes in percentage values of thymocyte subsets. Moreover, the activation of B cells, especially by LPS1, in the co-culture with thymocytes increased viability of SP CD4+, but not SP CD8+ thymocytes, which additionally favored SP CD4+ differentiation. Bieńkowska et al. have shown that providing two activatory signals using anti-CD3 and JAWS II cells (delivering co-stimulatory signal) shifted the differentiation of DP thymocytes into SP CD4+ thymocytes (Bieńkowska et al., 2014). In other study 7-fold increase in thymocyte numbers was observed in mice treated with superagonist (SA) anti-CD28 antibodies. The treatment induced proliferation of mature thymocytes, but did not accelerate differentiation of DP thymocytes (Legrand et al., 2006).

To examine the role of B cells in nTreg generation we followed a standardized protocol facilitating the assessment of sequential steps of nTreg generation based on the distribution of thymocyte subsets and maturation stage of nTregs as measured by CD25 and Foxp3 expression in SP CD4+ thymocytes. To follow the sequential steps of nTreg development, CD25 expression in SP CD4+ cells and subsequently Foxp3 expression in CD4+CD25+ cells have been investigated. The results revealed that the presence of B cells resulted in the induction of CD25 on SP CD4+ thymocytes and substantial maturation of nTregs (Foxp3 induction) (Fig. 3.8).

The main goal of our studies was to investigate the role of B cells in the generation of nTregs in *in vitro*. We hypothesize that B cells isolated from spleens can deliver both signals important to developing nTreg and in consequence induce their maturation. It was found that Tregs are induced by TCR signals below the threshold limit for negative selection and above the required affinities for conventional TCRs. nTreg selection seems to be directed by high affinity interaction of TCR with either self (Jordan et al., 2001; Kawahata et al., 2002) or foreign antigens (Pacholczyk et al., 2007). Thus, to prove our hypothesis, co-cultures of isolated splenic B cells with thymocytes containing nTreg precursors have been performed. According to the two-step model for nTreg differentiation (Burchill et al., 2008; Lio and Hsieh, 2008) developing nTregs (nTreg precursor) require co-stimulation signals from TCR/CD28 interactions with their ligands on B cells to induce the expression of CD25. It was documented that there was a significant reduction in the number of Tregs in thymus of $CD28^{-/-}$ mice and mice treated with anti-B7 antibodies (Tang et al., 2003; Lohr et al., 2004; Tai et al., 2005). Furthermore, markedly reduced numbers of peripheral CD4+CD25+ Tregs compartment in B7-1 and B7-2-deficient non-obese diabetic (NOD) mice was observed (Salomon et al., 2000). These observations support the importance of interaction between B7 and CD28 molecules in the nTreg generation. In our model the first step of nTreg development was accomplished by mere presence of B cells in co-cultures, that provided a weak signal to TCR. To strengthen this signal anti-CD3 activation step was further performed. Our results have shown that B cells can provide both signals required for generation of nTregs even without activation. The percentage of nTregs after anti-CD3 pre-activation increased 2-fold compared to previous co-culture activated by LPS1 (Fig. 3.6 and 3.15). This result is consistent with other findings showing that pre-activation of thymocytes by anti-CD3 before their culture with JAWS II cells was necessary for extensive generation of

nTregs (Bieńkowska et al., 2014). nTreg precursors need additional signal (for example from IL-2 or other cytokines or molecules) to induce the expression of Foxp3. In the aforementioned experiment non-activated B cells were not able to provide this signal to nTreg precursors. On the other hand, our result revealed that mere presence of B cells in co-culture can induce the expression of Foxp3 compared to thymocytes cultured alone (Ctrl) (Fig. 3.9). Additionally, B cell activation was crucial to induce Foxp3 expression in CD4+CD25+ thymocytes. Induction of Foxp3 requires an agonist self-peptide, and the frequency of nTreg cells that develop is directly proportional to the strength of the signal (Relland et al., 2009). Thus, activation of B cells used in the present study through TLR4 and TLR7 ligands increased the expression of co-stimulatory molecules, and in consequence increased the potential of B cells to generate nTregs. In addition, thymocytes : B cells ratio was pivotal for nTreg generation by B cells (Fig. 3.8). We have shown that the activation of B cells and/or increased number of B cells in co-cultures can be sufficient to induce Foxp3 expression and maturation of developing nTregs. These results clearly confirmed the role of B cells in the generation of nTregs by up-regulation of Foxp3 expression in developing nTregs (Fig. 3.9). Thus, activated B cells have the potential to generate nTregs by providing co-stimulatory signals to developing nTregs. These results are concordant with data published previously (Bieńkowska et al., 2014).

We showed that increased percentage of nTregs did not depend on TLR ligands used in the study (LPS and IMQ). These results suggest that the optimal conditions for nTreg generation *in vitro* depend on two factors: the presence of B cells (the percentage of nTregs increased with the increase of relative B cells number), and the B cells activation state (the percentage of nTregs increased in LPS and IMQ activated co-cultures). On the other hand, it was reported that many cells and molecules can induce the generation of Tregs, for instance the interaction of T cells with tolerogenic dendritic cells, anti-inflammatory cytokines, glucocorticoids, vitamins, or other suppressive molecules produced by pathogens (Bettelli et al., 2006). In our studies thymic nTreg was generated in the presence of splenic B cells activated by LPS and IMQ.

To elucidate if splenic B cells have the ability to induce the proliferation of generated nTreg, the proliferation of nTregs in various culture conditions has been investigated. We did not record any proliferation of nTregs in all control conditions (thymocytes cultured alone), what indicates that nTregs in control did not receive any signal

neither from thymic B cells nor from activators through TLRs and thereby they were unable to proliferate. This result is consistent with the results of Bieńkowska et al. who found that nTregs could not proliferate in control and in cultures activated with anti-CD3 only, whereas the highest proliferation was recorded when nTregs were provided with two signals (using anti-CD3 antibodies and JAWS II cell line as a source of co-stimulatory signal) (Bieńkowska et al., 2014). However, the proliferation of nTregs in co-culture of splenic B cells with thymocytes strongly depended on the ratio of B cells. nTregs revealed low level of proliferation in all culture conditions (thymocytes : B cells ratio 10:1). When the ratio of splenic B cells in co-culture with thymocytes has changed from 10:1 to 1:1, a small level of proliferation of nTregs was observed (data not shown because the number of events in the examined sample was not adequate for proper proliferation analysis, thereby we cannot show this result as a histogram).

To characterize the potential of B cells to generate nTregs *in vitro*, the expression of co-stimulatory molecules as well as MHC II in co-culture with thymocytes have been studied. We have shown that the expression of CD80 molecule on splenic B cells increased in co-culture with thymocytes compared to Ctrl, whereas the results of CD86, CD40, and MHC II molecules expression on splenic B cells were the opposite (Fig. 3.10 and 3.11). This observation should be interpreted in relation to the following facts. The affinity of CD86 on B cells to CD28 on T cells is 2-3-fold higher than that of CD80 (Collins et al., 2002), while the affinity of CTLA-4 to CD80 and CD86 is about 100-fold higher (depending on the ligand) than that of CD28 (Sansom et al., 2003). Moreover, CD86 is expressed constitutively and is rapidly up-regulated following T cell interaction with APCs, while CD80 is up-regulated at later stages of the immune response (Lenschow et al., 1996). Although CD28 is the preferred receptor for CD86, CTLA-4 binds mostly to CD80 (Pentcheva-Hoang et al., 2004; Evans et al., 2005). CD28 is constitutively expressed on naïve T cells, while CTLA-4 is not expressed constitutively and its expression is up-regulated upon T cell activation. For these reasons, we suggest that the down-regulation of some molecules and up-regulation of others on splenic B cells in co-culture with thymocytes compared to Ctrl may be due to interaction with their ligand/s on T cells able to transmit signal/s from B cell to T cell.

It has been suggested that nTregs require at least two signals for their generation, but the involvement of different molecules and cytokines in this process is still

controversial (Tang et al., 2003; Hsieh et al., 2006; Liston and Rudensky, 2007; Bayer et al., 2007; Moore et al., 2010; Bieńkowska et al., 2014; Lu et al., 2015).Thus, we have investigated the role of different molecules expressed by B cells on the generation of nTregs using molecule-blockade strategy by antagonist antibody. We have shown that the effect of B7 (CD80 and CD86) and MHC II molecules were more pronounced than that of CD40 molecule, and MHC II molecule has a pivotal role in the generation of nTregs (Fig. 3.12). We concluded that B cells can play a pivotal role in the generation of nTregs and can provide both signals needed for nTregs maturation. Additionally, B cells provide these signals through MHC II and co-stimulatory molecules, and the signal provided by CD80/CD86 is more efficient than signal provided by CD40. This result is consistent with previously published data (Salomon et al., 2000; Tang et al., 2003; Bour-Jordan et al., 2004; Tang et al., 2004; Lohr et al., 2004; Spence and Green, 2008; Bieńkowska et al., 2014).

We investigated the biological activity of nTregs generated from LPS1-activated co-cultures. CD4+ T cells were activated by anti-CD3/CD28 to induce proliferation. nTreg cells isolated from co-cultures effectively and significantly decreased activated CD4+ T cells. These results indicate that generated nTregs can exert suppressive activity against CD4+ T cells in *in vitro* conditions (Fig. 3.13). *In vitro* generated nTregs in can be used for the treatment of autoimmune diseases and prevention of transplant rejection (Chai et al., 2005; Shevach, 2006; Roncarolo and Battaglia, 2007; Haribhai et al., 2009; 2011; Lim et al., 2010; Qian et al., 2011; Nishikawa and Sakaguchi, 2014). However, results of other studies indicated that Tregs generated using different strategies in *in vitro* cultures were not always characterized by suppressive function and in many cases failed to suppress target cells (Prado et al., 2011). Additionally, other results showed that iTregs generated by activation with anti-CD3/CD28 antibodies differ from those generated by the physiological-like activation with antigen/APC (Zhao et al., 2014). Thus, different strategies of nTreg generation may give different results.

The pre-activation by anti-CD3 monoclonal antibody increased percentage of nTregs in thymocytes cultured alone compared to non-pre-activated thymocytes (1.10% and 0.26%, respectively). Additionally, the mere presence of splenic B cells in 72 h of co-culture with pre-activated thymocytes induced the increase of the percentage of nTregs indicating to the role of B cells in the generation of nTreg, while activation of B cells with pre-activation was of minor importance (Fig. 3.15). Collectively these

results revealed that the pre-activation with anti-CD3 (providing strong signal via TCR) could increase the percentage of nTregs when developing cells also receive second signal from B cells.
In this study, we introduce a novel method for nTregs generation by interaction with splenic B cells as APC.

3.2. Thymic B cells in the development of thymus-derived Tregs: preliminary studies

The results from the first section of the current study clearly confirmed the role of splenic B cells in the generation of nTregs in *in vitro* model. Additionally, these results revealed that B cells highly up-regulated MHC II as well as co-stimulatory molecules upon activation with TLR4 and TLR7 ligands resulting in increased number of nTregs generated in the presence of activated splenic B cells. It is considered that a very small number of B cells reside in the thymus and their contribution to nTreg generation was neglected in the experiments presented in the first section. To answer the question if thymic B cells play a role in the generation of nTregs we performed the following examinations:

1/ The percentage of nTregs in thymic cells cultured in the presence of LPS and IMQ.

2/ The percentage of thymic B cells in control medium and in LPS- and IMQ-conditioned medium.

3/ The expression of MHC II and co-stimulatory molecules on thymic B cells cultured in LPS- and IMQ-conditioned medium.

3.2.1. Role of thymic B cells in the generation of thymus-derived Tregs and Foxp3 expression *in vitro*

There is a small population of B cells in thymus (between $0.10 - 0.50\%$) (Frommer and Waisman, 2010; Perera et al., 2013) and their function and origin are still controversial. There are two suggested origins of thymic B cells. The first group has suggested that thymic B cells develop in the thymus from thymic B-cell progenitors and differ from recirculating peripheral B cells (Hashimoto et al., 2002; Perera et al., 2013; Perera and Huang, 2015). Second group argued that thymus contains mature B cells and the migration of peripheral B cells to the thymus might

increase several fold in certain pathological situations such as thymic lymphoma (Michie and Rouse, 1991) and some murine models of autoimmune diseases (Hart and Zan-Bar, 1991). Activation of thymic B cells with LPS and IMQ constitutes did not change the percentage of nTregs in the 24 h culture and increased in 72 h culture compared to non-activated controls (Fig. 3.17). Activation by LPS and IMQ did not change the percentage of nTreg regardless of duration of the culture. It is clearly depicted that the percentage of nTregs in 72 h culture is maintained at the same level as after 24 h demonstrating that B cell activation results from their increased survival.

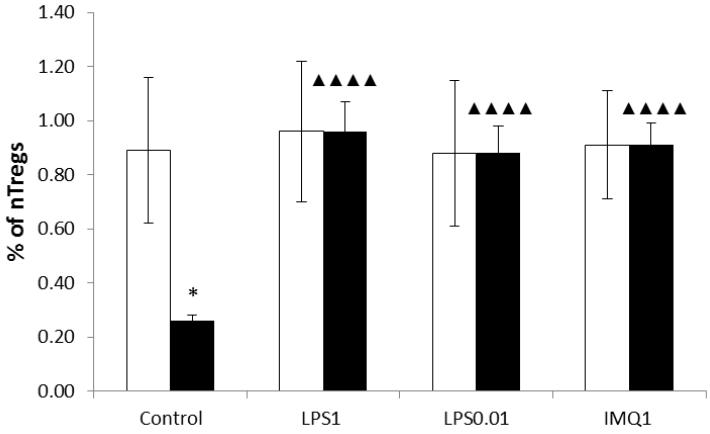


Figure 3.17:— The percentage of CD4+Foxp3+ thymocytes in the culture of thymus-derived cells. The experiments have been performed in triplicates. The ells were analyzed after 24h (white bars) and 72h (black bars) of *in vitro* culture. The significance values were indicated as▲ between Control (non-activated) and activated cells in 24 or 72 h cultures, and * between 24 and 72 h cultures. Control (non-activated cells), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001, and similarly for ▲).

The expression of Foxp3 was not changed upon activation by LPS and IMQ, with the exception of IMQ-supplemented medium, which caused the statistically significant up-regulation Foxp3 (Fig. 3.18).

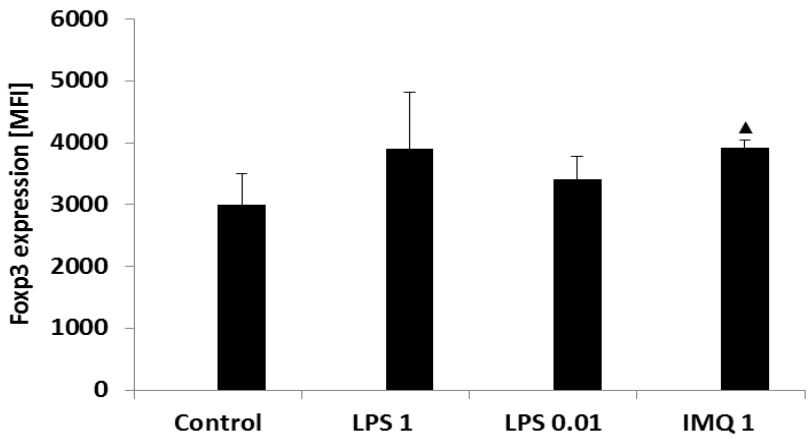


Figure 3.18:— The expression of Foxp3 molecule in nTreg cells in different conditions of thymus-derived cells.
The experiments have been performed in triplicates. The cells were analyzed after 72h of *in vitro* culture. The significance values were indicated as ▲ between Control (non-activated cells) and activated cells. Control (non-activated thymocytes), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$).

Summary:

1. The percentage of nTregs of control cultures decreased in time-dependent manner.
2. The percentage of nTregs in 24 h culture of thymocytes of LPS and IMQ-conditioned medium was not changed.
3. LPS and IMQ-mediated activation counteracted the decrease of nTregs in 72 h cultures maintaining the level observed in 24 h control culture by supporting their survival.
4. LPS and IMQ-supplemented medium did not substantially influence the expression of Foxp3 in nTregs.

3.2.2. B cells content in the culture of thymus-derived Tregs of LPS and IMQ-supplemented medium

In physiological conditions B cells represent approximately 0.50% of the thymic cellularity. Although their absolute number may exceed that of thymic dendritic cells, their role as antigen-presenting cells for central tolerance induction is not well understood (Yamano et al., 2015b). It was estimated that apart from the

release of about 1×10^6 T cells, each day the thymus exports around 3×10^4 of B cells (Akashi et al., 2000), which is a good evidence suggesting that part of the thymic B cells differentiate within the thymus. The percentage of B cells was strongly increased of our experimental conditions (LPS and IMQ-supplemented medium). Increased percentage of B cells was observed in 72 h cultures, which suggests that both activators induced proliferation. This suggestion is additionally confirmed by the fact that the increase of B cells depended on the concentration of the activator (namely of LPS) (Fig. 3.19).

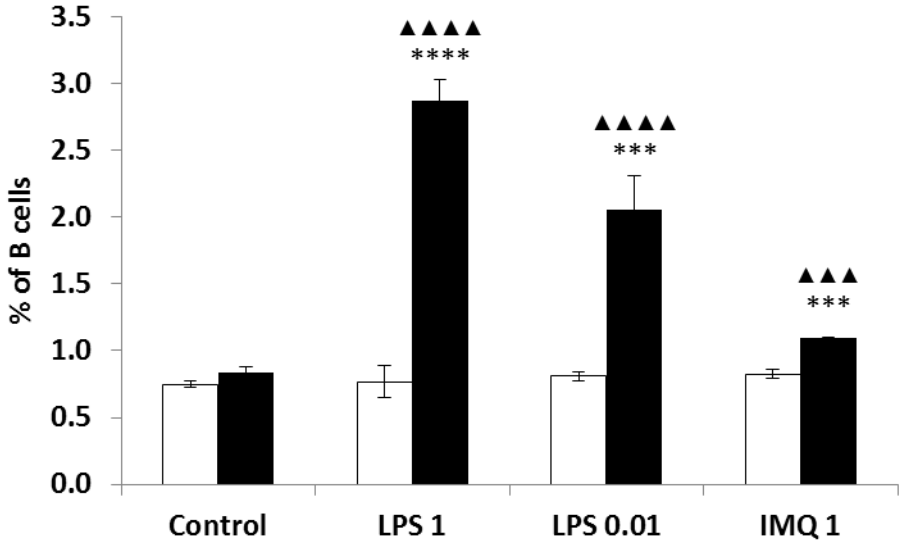


Figure 3.19:— The percentage of B cells in the culture of thymus-derived cells.
The experiments have been performed in triplicates. The cells were analyzed after 24 h (white bars) and 72 h (black bars) of *in vitro* culture.
The significance values were indicated as ▲ between Control (non-activated) and activated cells in 24 or 72 h cultures, and * between 24 and 72 h cultures. Control (non-activated thymocytes), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for *).

Summary:

1. LPS and IMQ supplementation resulted in increased percentage of B cells suggesting activator-induced proliferation of thymic derived B cells.

3.2.3. Expression of co-stimulatory molecules and MHC II on thymic B cells

We have investigated the expression of MHC II and co-stimulatory molecules on thymic B cells upon activation by LPS and IMQ. Unlike splenic B cells, the percentage of thymic B cells expressing CD80 and CD86 increased in LPS1- and IMQ1-activated 72 h cultures compared to 24 h (Fig. 3.20 A and B). Considering a

small number of thymic B cells, the concentrations of activators, LPS and IMQ, were high enough to saturate TLR4 and TLR7 maintaining the increased expression of molecules throughout the culture. The lower concentration of LPS was not effective in increasing the percentage of CD80 and CD86-positive B cells. LPS1 stimulation increased the percentage of CD86+ B cells in a time-dependent manner, whereas IMQ1 stimulation increased the percentage of CD86+ B cells only in 72 h cultures (Fig. 3.20 B). The percentage of CD40+ B cells was slightly increased upon activation with LPS1 and significantly decreased upon activation with LPS0.01 and IMQ1 in 24 h cultures (Fig. 3.20 C). LPS at higher concentration induced the increase of the percentage of MHC II+ thymic B cells in 72 h cultures while decreasing the percentage of MHC II+ B cells upon activation by the lower concentration of LPS and IMQ1 (Fig. 3.20 D).

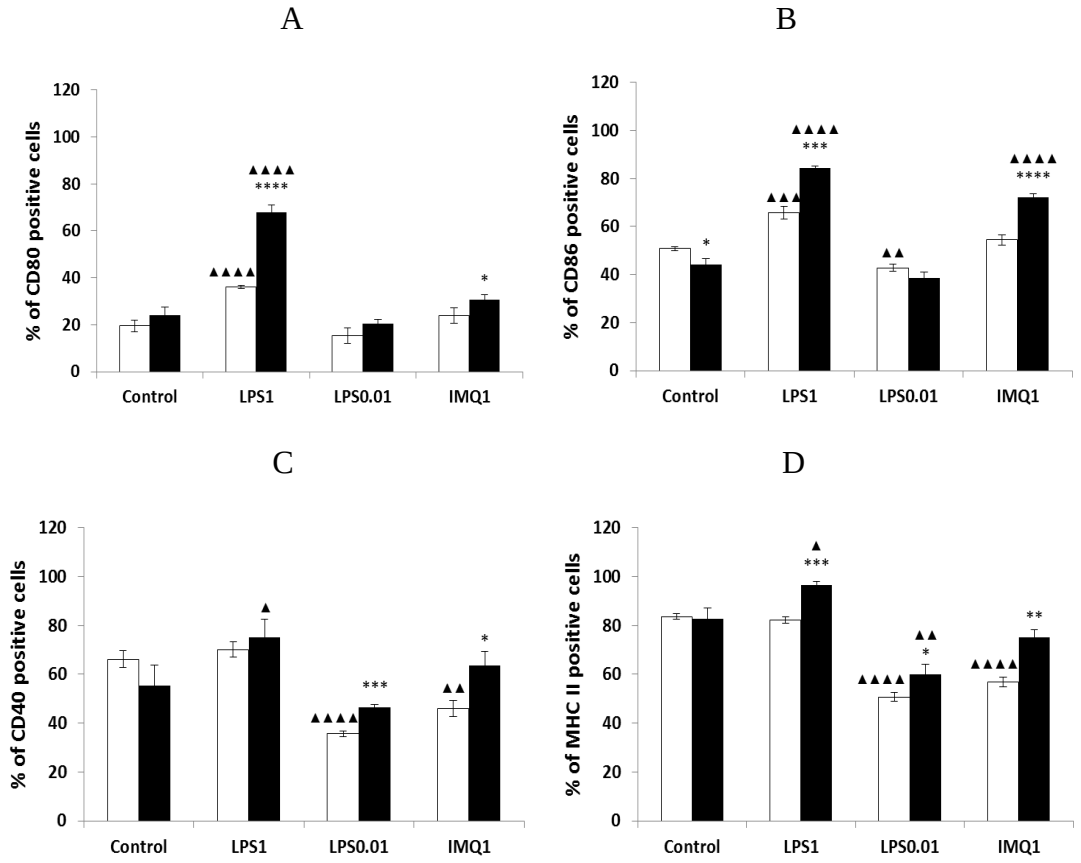


Figure 3.20:— The effect of LPS and IMQ on the percentage of thymic B cells positive for MHC II and co-stimulatory molecules. CD80 (A), CD86 (B), CD40 (C), and MHC II (D) *in vitro*.
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as▲ between Control (non-activated) and activated cells in 24 or 72 h cultures, and * between 24 and 72 h cultures. Control (non-activated cells), LPS1 (1μg/ml), LPS0.01 (0.01μg/ml), and IMQ1 (1μg/ml). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ▲).

CD80, CD86, CD40, and MHC II expression on thymic B cells is presented on Fig. 3.21. Unlike the percentage of thymic B cells positive for these molecules, the expression of MHC II and co-stimulatory molecules in B cells increased after 24 h cultures compared to 72 h cultures (except the expression of MHC II in IMQ1-activated culture/72 h). LPS1 and IMQ1 stimulation significantly increased the expression of CD80 in both culture durations as compared to control (Fig. 3.21 A), however, the up-regulations expression was inversely dependent on the time of culture. Similar correlations have been observed for the expression of CD86, CD40 and MHC II upon activation by LPS1 and IMQ1 (with the exception of MHC II for IMQ1). The expression of CD86 increased upon activation by LPS1 and IMQ1 compared to control, and was also inversely dependent on time (Fig. 3.21 B). LPS1 and IMQ1 stimulation induced up-regulation of CD40 and MHC II in a similar inversely time-dependent manner (Fig. 3.21 C and D).The diminished expression of the majority of molecules in 72 h cultures with the simultaneous increase of the percentage of positive B cells for these molecules point on the induction of investigated markers on negative B cells upon activation followed by the retarded up-regulation of their expression.

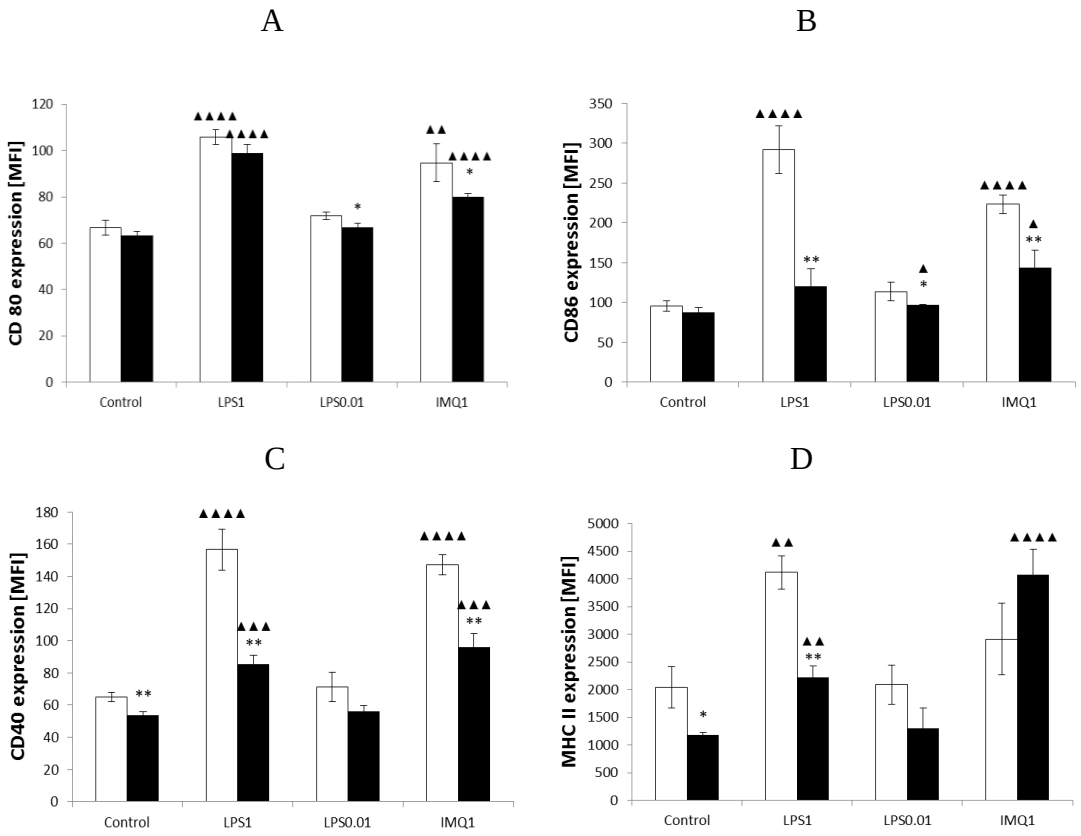


Figure 3.21:— The expression of co-stimulatory molecules. CD80 (A); CD86 (B); CD40 (C); and MHC II (D) on LPS and IMQ activated thymic B cells.
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as▲ between Control (non-activated) and activated cells in 24 or 72 h cultures, and * between 24 and 72 h cultures. Control (non-activated cells), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). The levels of probability were indicated as follows (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001, and similarly for ▲).

Summary:

1. LPS and IMQ activation resulted in the up-regulation of CD80, CD86, CD40, and MHC II on thymic B cells.
2. The up-regulation of CD80, CD86, CD40, and MHC II correlated with the increase of the percentage of B cells-positive for these molecules upon activation by LPS1.

Discussion

In this section, we have focused on the possible role of thymus-derived B cells in the generation of nTregs.

Thymus contains a small population of B cells that colocalize with dendritic cells and medullary thymic epithelial cells in the thymic medulla (Perera et al., 2013; Walters et al., 2014). The development and functional significance of these cells are

controversial. It was documented that the majority of thymic B cells are B1-cells (Inaba et al., 1990; Sugihara et al., 2000; Ceredig, 2002). B cells are considered inefficient in presenting antigens via nonspecific uptake, however, capture and internalization of cognate antigens with subsequent presentation is very efficient (Pierce et al., 1988; Lanzavecchia, 1990). Thus, for a particular antigen B cells specific to that antigen could be the most efficient antigen-presenting cells on a per cell basis.

In our experimental settings thymic B cells did not influence the generation of nTregs, but rather maintain their viability when activated by both studied activators (LPS or IMQ). They revealed the typical characteristics of antigen-presenting cells when activated by LPS and IMQ, which up-regulated MHC II and co-stimulatory molecules expression. This result differed from the result published by others who found that thymic B cells did not respond to stimulation by LPS or IL-4 and expressed low level of MHC II upon activation (Inaba et al., 1990). This discrepancy may result from the differences in experimental model.

It was documented that thymic B cells have a pivotal role in the generation of nTregs (Walters et al., 2014). In addition, thymic B cells are involved in negative selection of auto-reactive CD4+ thymocytes whereas nTregs can spare from this process thereby the percentage of nTregs was increased (Frommer and Waisman, 2010). In addition to the role of thymic B cells in the generation and/or selection of nTregs (Frommer and Waisman, 2010; Perera et al., 2013; Walters et al., 2014; Lu et al., 2015), we have shown that activated thymic B cells maintained the viability of thymic nTregs as confirmed by increased percentage of nTregs compared to non-activated 72 h culture (Fig. 3.17). Our results documented the role of thymic B cells in supporting the viability of thymic Tregs. Consistent with this result, it was documented that the generation of nTregs in thymus is accomplished in two steps. In the first step, thymic B cells promote the generation of thymic pre-nTreg thymocytes from CD4 single positive thymocytes. In the second step, thymic B cells directly promote the proliferation of thymic Treg cells both *in vivo* and *in vitro* in MHC II-dependent manner with a minimal-role for the co-stimulatory molecules CD40 and CD80/86 (Lu et al., 2015).

Our results revealed that unlike splenic B cells, the percentage of thymic B cells expressing both MHC II and co-stimulatory molecules (but not the expression of these molecules) up-regulated in 24 h cultures and continued to increase during incubation upon activation by T-independent stimuli (LPS and IMQ) compared to control, indicating that thymic and splenic B cells derive from different origins. In addition, thymic B cells maturated after 24 h of activation and served as mature cells during further incubation (Fig. 3.20). Thymic B cells may proliferate upon activation via TLR4 and TLR7 because the percentage of B cells in thymus significantly increased in activated cultures compared to non-activated cultures/72 h (Fig. 3.19). This result was consistent with previously published data (Inaba et al., 1990).

3.3. Effect of dexamethasone on the generation of thymus-derived Tregs in the co-culture of thymocytes and splenic B cells

Glucocorticoids exert multiple effects on immune cells, such as induction of apoptosis and up- or down-regulation of genes expression (Elenkov, 2004) including Foxp3 of Tregs (Prado et al., 2011). Glucocorticoids are known for their immunosuppressive effects, and are widely used as potent immunosuppressive agents in different immune diseases and experimental settings (Madden and Felten, 1995; Tarcic et al., 1998; Yoshimura et al., 2001).

Dexamethasone (Dex) is a synthetic glucocorticoid widely used as a potent suppressor of inflammatory response. It has been documented that tolerogenic dendritic cells contribute to the induction of Tregs. Treatment of human monocyte-derived DC or mouse bone marrow-derived DC with Dex conditioned these cells for tolerogenic phenotype and activity to induce T regulatory cells (Maldonado and von Andrian, 2010). Ligand binding to the glucocorticoid receptor regulates DC activation through nuclear glucocorticoid response elements that negatively regulate promoters for genes involved in canonical NF-κB pathway, inflammatory cytokines, chemokines, their receptors and antigen presentation molecules (Leung and Bloom, 2003). In addition to repressing DC maturation, Dex also induces expression of multiple anti-inflammatory genes and chemoattractants, including IL-10, GITRL, IDO, CCL2 (MCP-1), CCL8 (MCP-2), CCR2, CCL9 (MIP-1c) and CCLl2 (MIP-2) (Roca et al., 2007; Grohmann et al., 2007). This impairs the ability of DCs to migrate and provokes them to aquire a tolerogenic phenotype capable of directing naïve T cells to express CD25 and Foxp3.

On the other hand, the inhibitory effect of glucocorticoids on NF-κB pathway likely plays a key role in the conversion of DCs to the tolerogenic phenotype. Also, inhibition of NF-κB or IKKβ by small molecule antagonists generates tolerogenic DCs able to stimulate Foxp3+CD25+ Tregs and alleviate symptoms of EAE, heart allograft rejection, and intestinal bowel disease (Buckland et al., 2006; Iruretagoyena et al., 2006; Zhang et al., 2008; Buckland and Lombardi, 2009; Cong et al., 2009). Moreover, tolerogenic DCs are able to promote differentiation and proliferation of regulatory T cells (Huang et al.,2010), and can be generated *ex vivo* from peripheral blood monocytes modulated by different approaches, including conditioning with pharmacological agents such as Dex and rapamycin (Matsue et al., 2002; Unger et al., 2009).

The aim of the studies presented in this section was to investigate whether dexamethasone can induce a tolerogenic potential of B cells similarly to its effect on dendritic cells and facilitate the generation of nTregs from thymic precursors. In order to complete this part of the study the following tasks have been performed:

1/ Examination of the role of Dex on the expression of MHC II and co-stimulatory molecules (CD80, CD86, and CD40) on splenic B cells upon activation by LPS and IMQ.

2/ Investigation of the role of Dex on the distribution of the main thymocyte subsets.

3/ Determination of the effect of Dex on the generation of thymus-derived Treg cells in co-culture with splenic B cells.

4/ Analysis of the effect of Dex on the expression of MHC II and co-stimulatory molecules on splenic B cells in co-culture with thymocytes.

5/ Evaluation of Dex-induced B cells apoptosis in different culture conditions.

3.3.1. The effect of dexamethasone on the expression of MHC II and co-stimulatory molecules on splenic B cells

As it was shown previously, the percentage of splenic B cells expressing CD80, CD86, CD40, and MHC II was increased upon activation by LPS and IMQ. The percentage of B cells expressing MHC II and co-stimulatory molecules was decreased significantly in the presence of Dex (Fig. 3.22, 3.23, 3.24, and 3.25).

The percentage of CD80+ splenic B cells activated by LPS and IMQ in the presence of Dex is presented on Figure 3.22.

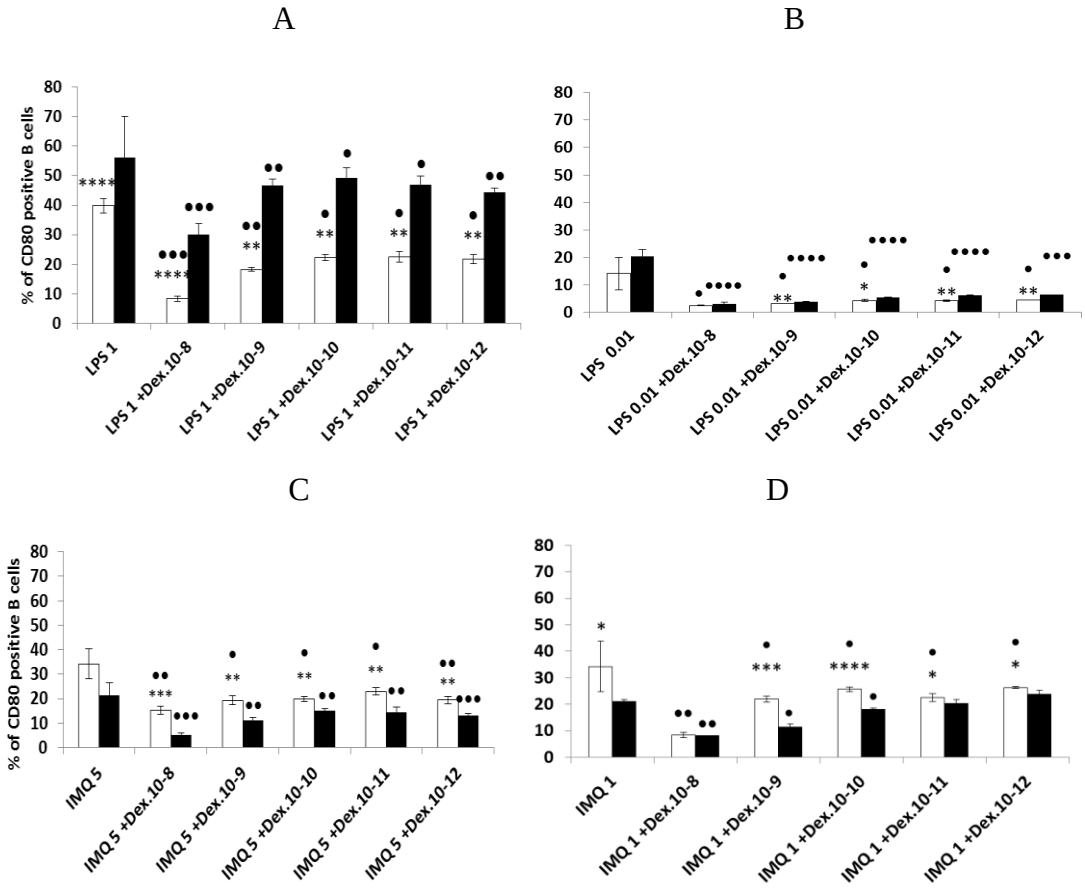


Figure 3.22:— The effect of dexamethasone on the percentage of CD80+ splenic B cells activated with LPS and IMQ. LPS1 (1µg/ml) (A), LPS0.01 (0.01µg/ml) (B), IMQ5 (5µg/ml) (C), and IMQ1 (1µg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ●).

Dex decreased the percentages of CD80+ B cells both in LPS and IMQ activated cultures. The decrease was inversely proportional to the level of activation of B cells (by signals delivered from a particular TLR) characterized by the percentage of CD80+ splenic B cells in activated cultures without Dex. Most potent decrease of the percentage of CD80+ B cells was obtained in the presence of the highest concentration of Dex (10^{-8}M). This concentration induced 5-fold and 1-fold decrease of CD80+ B cells in LPS1-activated cultures after 24 and 72 h of incubation, respectively. On the other hand, the same concentration decreased this percentage 6-fold and 7-fold in LPS0.01-activated cultures for 24 and 72 h, respectively (Fig. 3.22

A and B). The same effect was observed in IMQ-activated cultures. The decrease was time dependent for all doses of activators (Fig. 3.22 C and D).

Dex decreased the percentages of CD86+ B cells both in LPS and IMQ activated cultures. In LPS-activated cultures, the decrease was inversely proportional to the level of activation of B cells and proportional to the concentration of Dex, while the decrease of the percentage was similar in both IMQ-activated cultures. The decrease was time dependent for all doses of activators (Fig. 3.23 A, B, C and D).

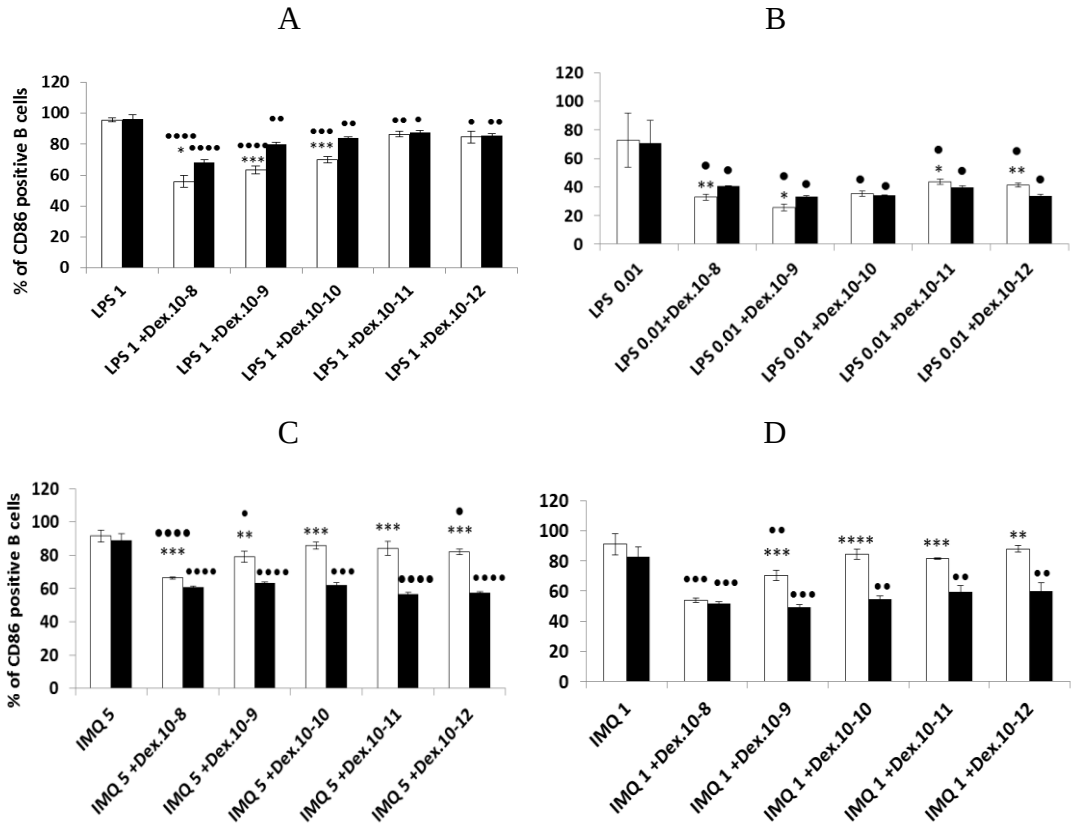


Figure 3.23:— The effect of dexamethasone on the percentage of CD86+ splenic B cells activated with LPS and IMQ. LPS1 (1µg/ml) (A), LPS0.01 (0.01µg/ml) (B), IMQ5 (5µg/ml) (C), and IMQ1 (1µg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001, and similarly for ●).

Dex decreased the percentages of CD40+ B cells both in LPS and IMQ activated cultures. The decrease was inversely proportional to the level of activation of B cells. This dependence was more pronounced in LPS-activated B cell cultures (Fig. 3.24 A and B). Low concentration of LPS decreased the sensitivity to Dex in 72 h culture resulting in an increase of CD86+ B cells compared to 24 h culture and diminution of

the difference in the percentage of positive B cells compared to appropriate activated control culture without Dex. This effect was not observed in cultures activated by high concentration of LPS as well as both concentrations of IMQ. The effect of Dex was dose- and duration-dependent for LPS-activated cultures, however, it was neither dose of activator nor duration-dependent for IMQ-activated cultures (Fig. 3.24 A, B, C and D).

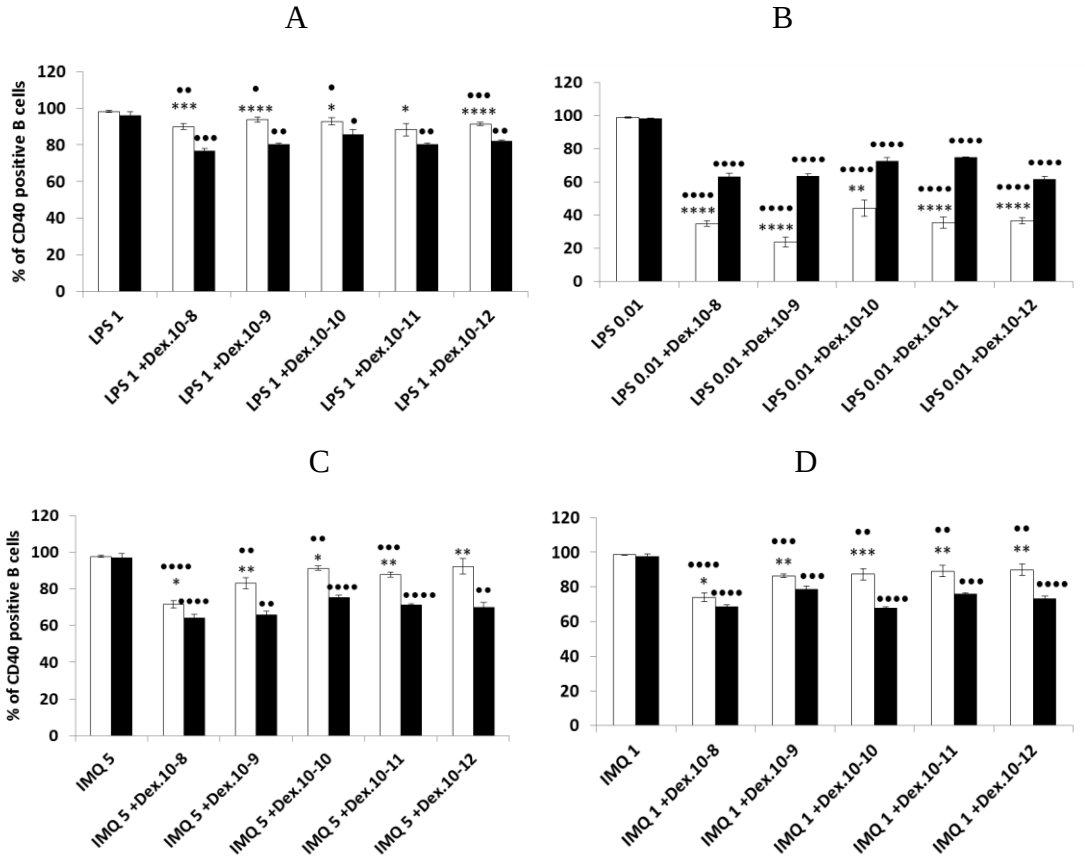


Figure 3.24:— The effect of dexamethasone on the percentage of CD40+ splenic B cells activated with LPS and IMQ. LPS1 (1µg/ml) (A), LPS0.01 (0.01µg/ml) (B), IMQ5 (5µg/ml) (C), and IMQ1 (1µg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ●).

Dex decreased the percentages of MHC II+ B cells both in LPS- and IMQ-activated cultures. The suppressive effect of Dex on the percentage of MHC II+ B cells was substantially weaker than on co-stimulatory molecules (Fig. 3.25 A, B, C and D).

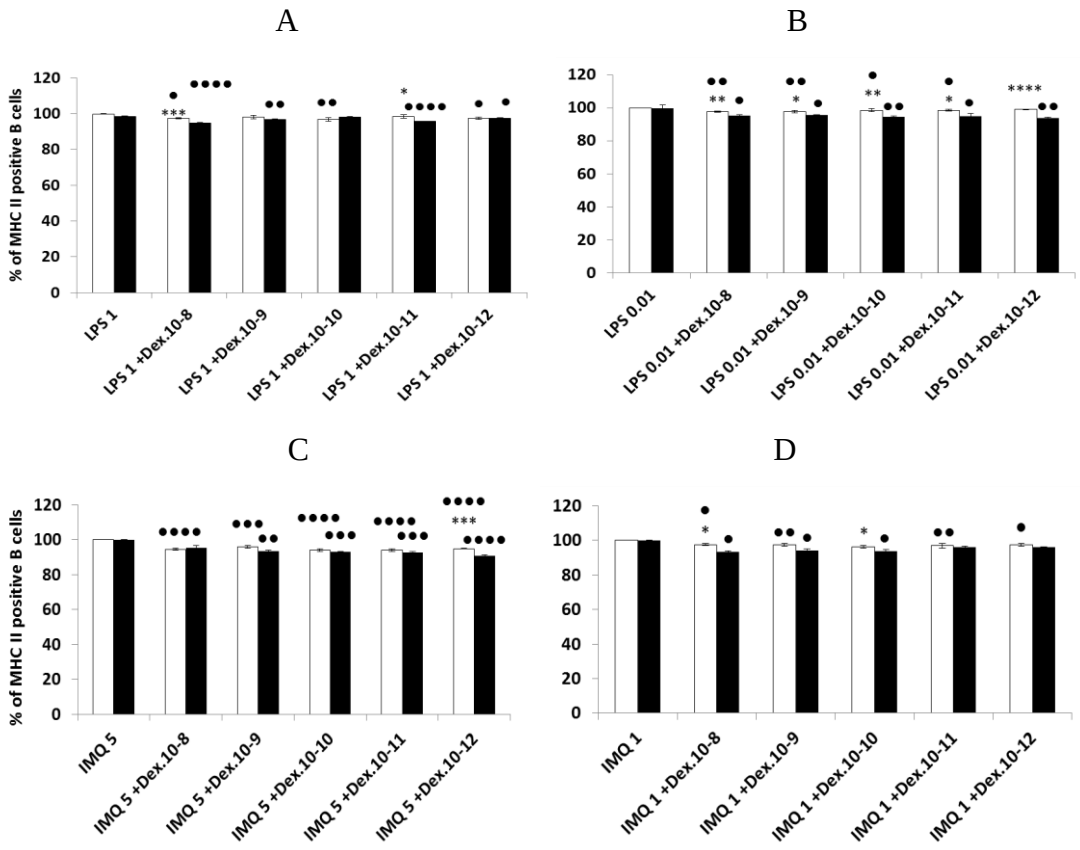


Figure 3.25:— The effect of dexamethasone on the percentage of MHC II+ splenic B cells activated with LPS and IMQ. LPS1 (1µg/ml) (A), LPS0.01 (0.01µg/ml) (B), IMQ5 (5µg/ml) (C), and IMQ1 (1µg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ●).

The expression of CD80, CD86, CD40 and MHC II molecules have been investigated. In contrary to its role as suppressive agent decreasing the percentage of CD80 positive B cells, some concentrations of Dex (10^{-9} to 10^{-12} M) up-regulated CD80 expression independently from the concentrations of activators (LPS or IMQ), especially in 72 h cultures (Fig. 3.26). 10^{-8} M concentration of Dex decreased the expression of CD80 molecule in 24 h cultures activated by LPS and IMQ. We suggest that the apparent increase of CD80 expression resulted from the difference in the expression of this molecule among the population of cultured B cells: the expression of CD80 was completely inhibited on B cells expressing low level of this molecule (the percentage of B cells positive for CD80 was decreased by Dex), leaving B cells, which originally showed higher expression of CD80.

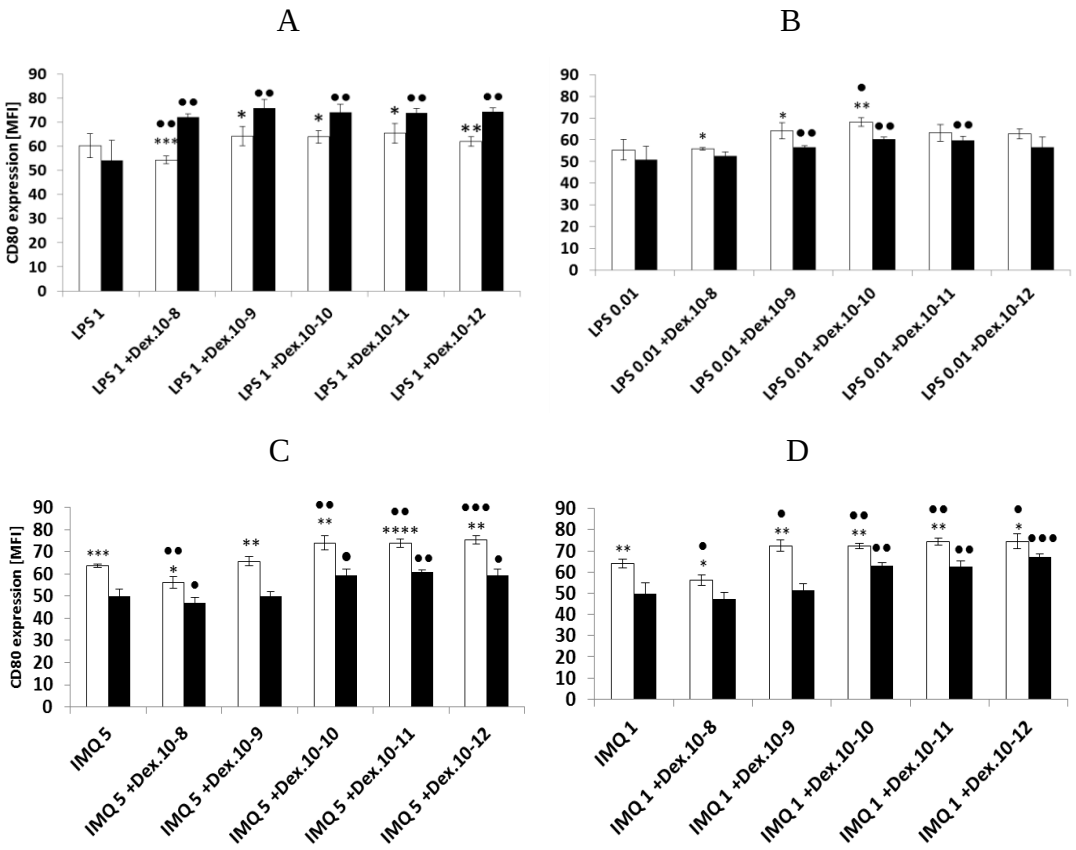


Figure 3.26:— The effect of dexamethasone on the expression of CD80 molecule on splenic B cells activated with LPS and IMQ. LPS1 (1μg/ml) (A), LPS0.01 (0.01μg/ml) (B), IMQ5 (5μg/ml) (C), and IMQ1 (1μg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ●).

The suppressive effect of Dex was mainly observed at the higher concentration of LPS in 24 h cultures, and the suppressive effect depended on Dex concentrations. In 72 h LPS high-activated cultures, two concentrations of Dex (10^{-9} and 10^{-10} M) significantly increased the expression of CD86 molecule compared to activated sample (Fig. 3.27 A). Low concentration of LPS combined with low concentrations of Dex (10^{-10}, 10^{-11}, and 10^{-12} M) significantly increased the expression of this molecule (Fig. 3.27 B). In IMQ-activated cultures, the suppressive effect of Dex was observed only in 24 h cultures in the medium containing the highest concentration of Dex (Fig. 3.27 C and D).

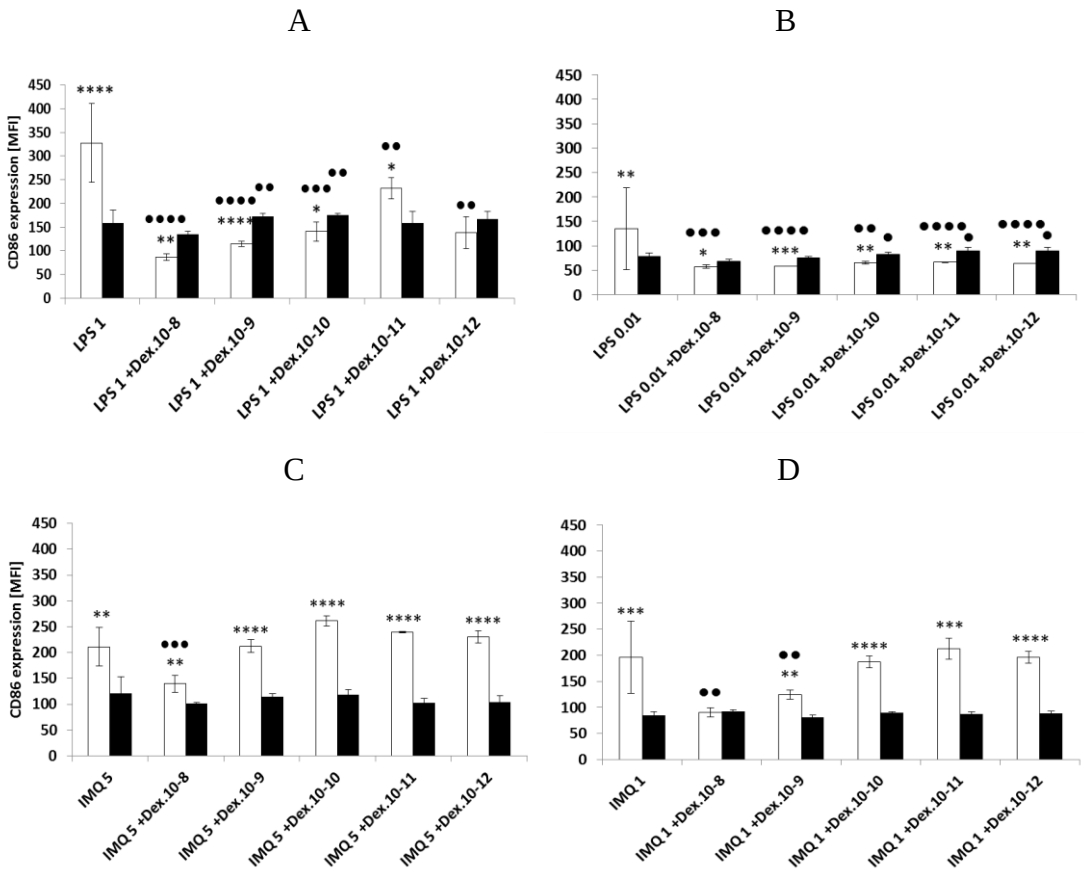


Figure 3.27:— The effect of dexamethasone on the expression of CD86 molecule on splenic B cells activated with LPS and IMQ. LPS1 (1µg/ml) (A), LPS0.01 (0.01µg/ml) (B), IMQ5 (5µg/ml) (C), and IMQ1 (1µg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ●).

Dex suppressed the expression of CD40 on B cells in the majority of cultures independently on the type of activator and duration of culture, and did not affect the expression of this molecule in 24 h cultures upon activation by IMQ.

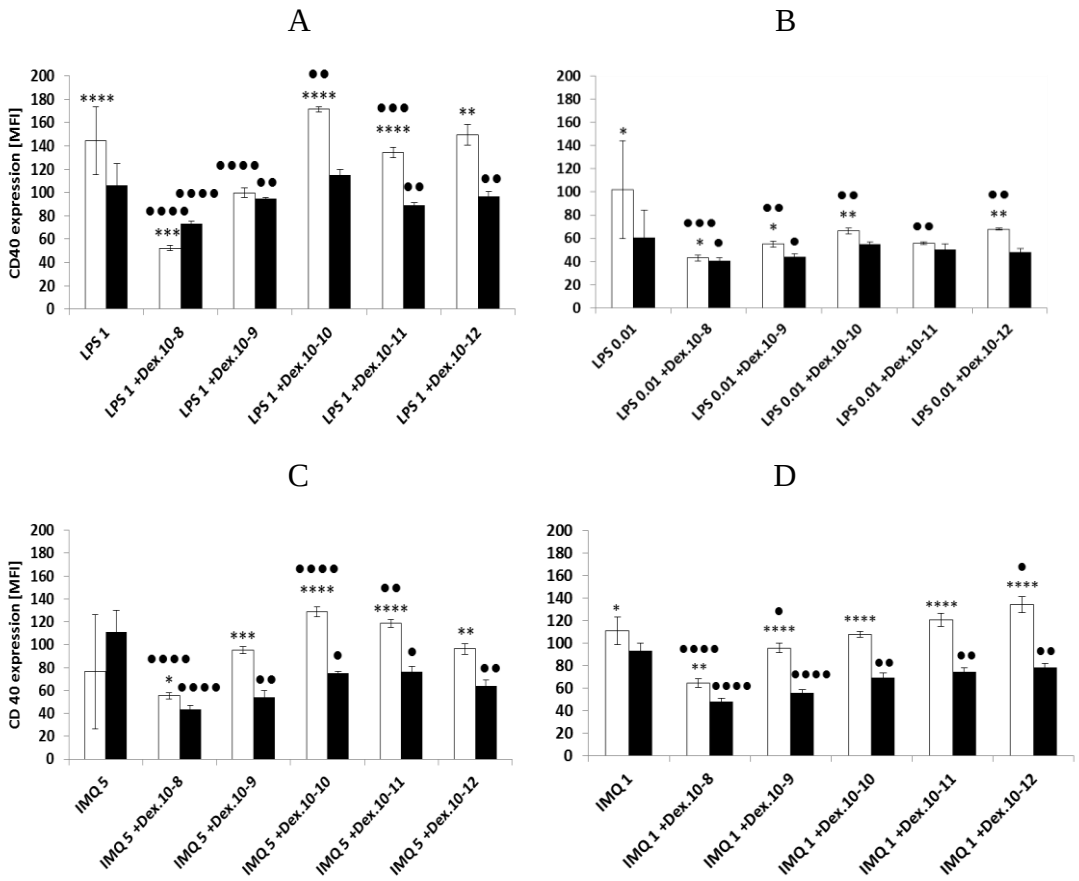


Figure 3.28:— The effect of dexamethasone on the expression of CD40 molecule on splenic B cells activated with LPS and IMQ. LPS1 (1µg/ml) (A), LPS0.01 (0.01µg/ml) (B), IMQ5 (5µg/ml) (C), and IMQ1 (1µg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001, and similarly for ●).

While all concentrations of Dex down-regulated CD40 expression in 72 h cultures when added to IMQ-activated cultures, two concentrations of Dex (10^{-10} and 10^{-11} M) for IMQ high-activated culture and one concentration of Dex (10^{-12} M) for IMQ low-activated culture up-regulated this molecule/ 24 h (Fig. 3.28C and D). We suggest that the strength of signal delivered to B cells via TLR4/LPS or TLR7/IMQ play a crucial role in antagonizing the suppressive effect of Dex in 24 h cultures, however, this effect did not sustain for long period of incubation (for 72 h cultures).

Dex strongly inhibited of MHC II expression independently from the concentrations of activators and duration of culture.

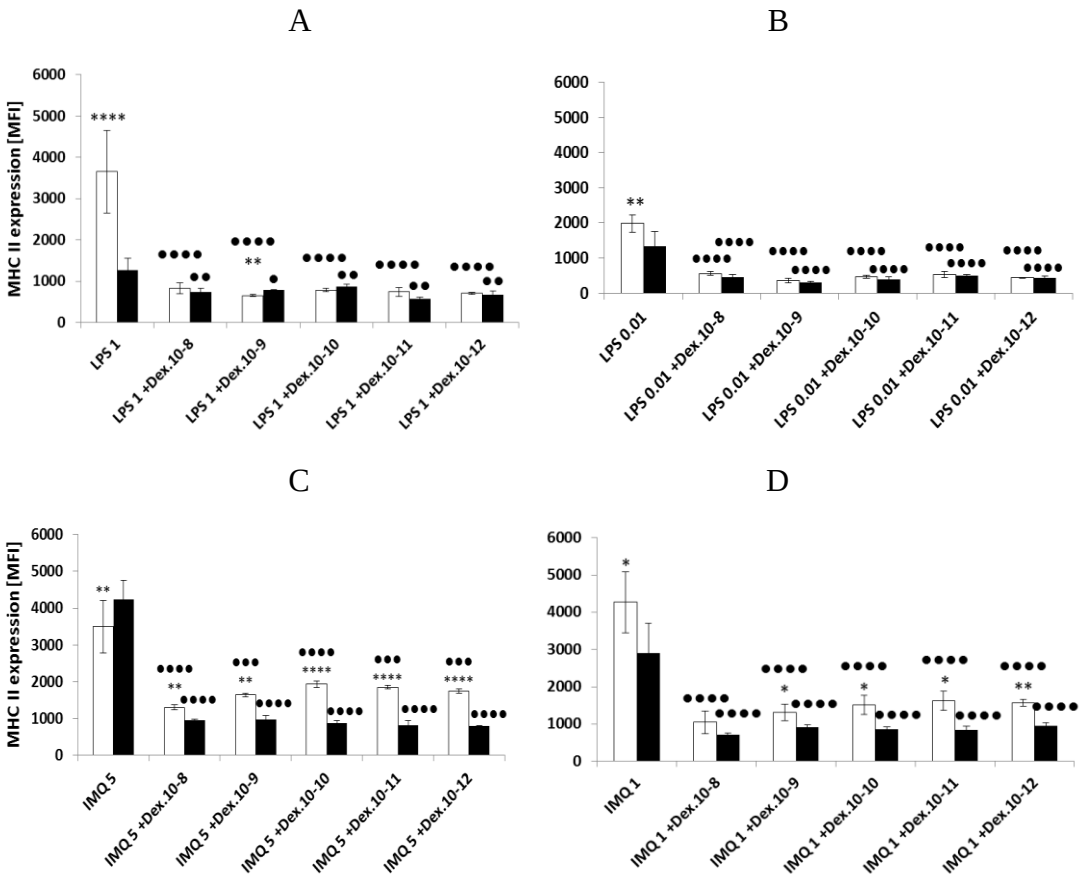


Figure 3.29:— The effect of dexamethasone on the expression of MHC II molecule on splenic B cells activated with LPS and IMQ. LPS1 (1µg/ml) (A), LPS0.01 (0.01µg/ml) (B), IMQ5 (5µg/ml) (C), and IMQ1 (1µg/ml) (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001, and similarly for ●).

Summary:

1. Dexamethasone suppressed the expression of co-stimulatory molecules that was observed as decrease of B cells expressing particular molecules and decreased expression level of molecules of interest. CD80 and CD86 expression was the most affected, and was less potent for CD40.
2. MHC II expression level was strongly suppressed by dexamethasone independently of dexamethasone concentrations and duration of culture without substantial effect on the percentage of MHC II+ B cells.

3.3.2. The effect of dexamethasone on the distribution of the main thymocyte subsets

The effect of dexamethasone on the distribution of the main thymocyte subsets in co-culture with splenic B cells (Thymocytes : B cells ratio 10:1) was examined (Fig. 3.30). The distribution of the main thymocyte subsets was not affected by dexamethasone.

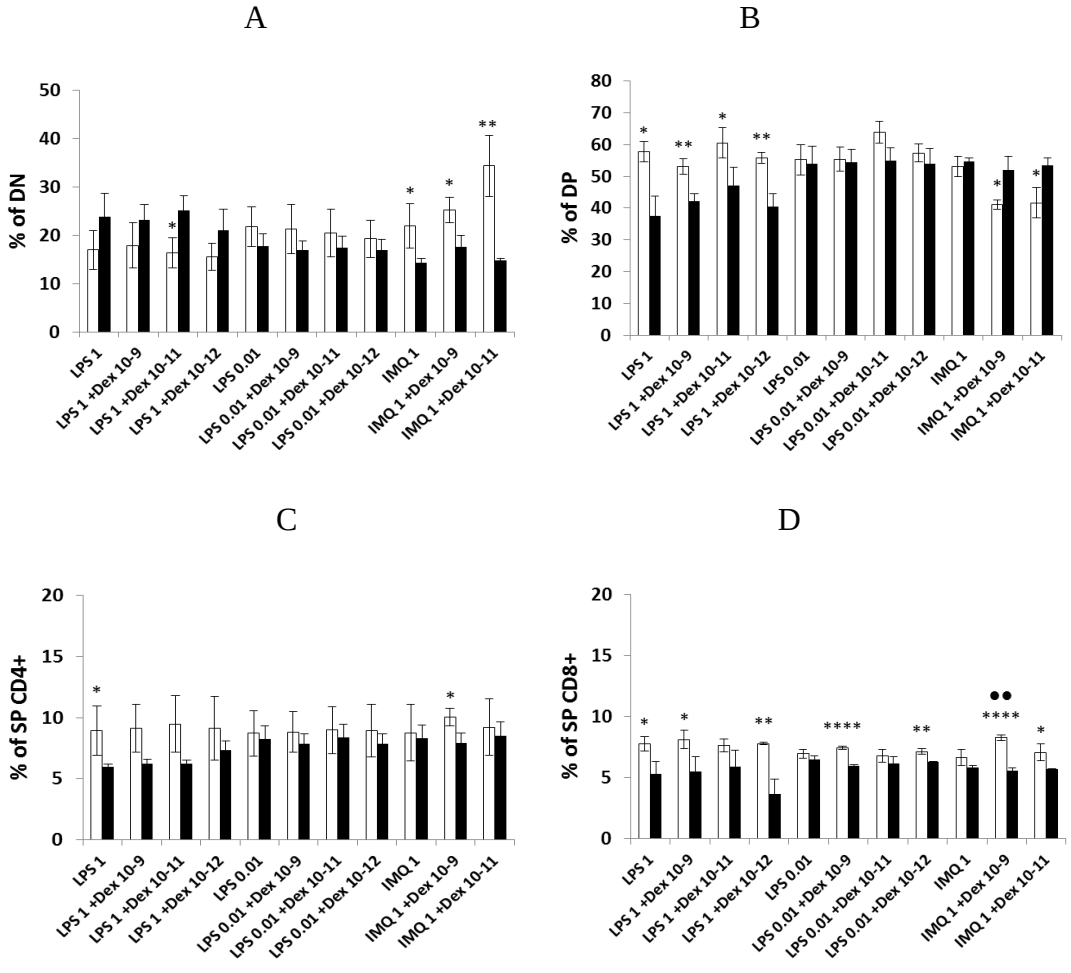


Figure 3.30:— The effect of dexamethasone on the distribution of thymocyte subsets in co-culture with splenic B cells (thymocytes : B cells ratio 10:1). DN (A), DP (B), SP CD4+ (C), and SP CD8+ (D).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-9} (10^{-9} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ●).

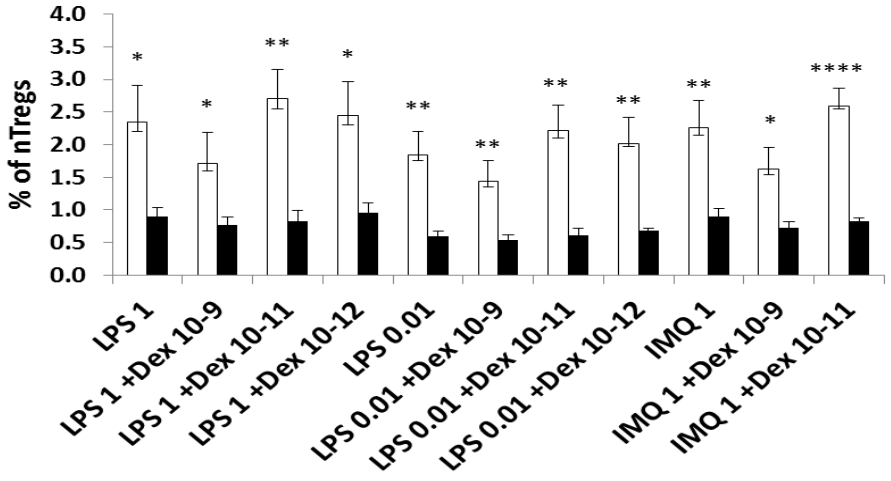
Summary:

1. Dexamethasone did not affect the distribution of the main thymocyte subsets in co-cultures of thymocytes and B cells activated by LPS and IMQ.

3.3.3. Effect of dexamethasone on the generation of thymus-derived Tregs in co-culture with splenic B cells

The presence of Dex in co-cultures (Thymocytes : B cells ratio 10:1) did not affect the percentage of nTregs regardless of the duration of culture. The difference in the percentage of nTregs between 24 h and 72 h cultures were maintained independently of the presence of dexamethasone in culture medium (Fig. 3.31 A).

A



B

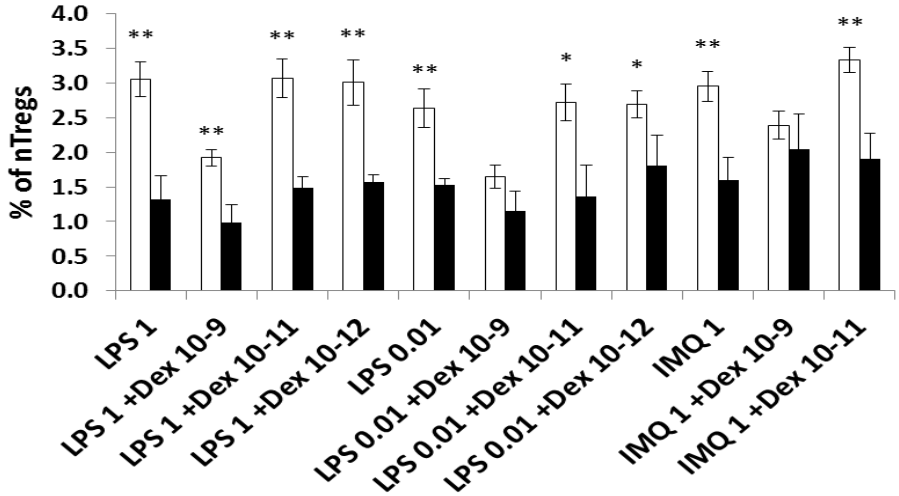


Figure 3.31:— The effect of dexamethasone on the percentage of CD4+Foxp3+ (nTreg) cells in co-culture with splenic B cells. (A) (Thy : B ratio=10:1), and (B) (Thy : B ratio=1:1).
The experiments have been performed in triplicates. The cells were analyzed after 24 (white bars) and 72 (black bars) hours of *in vitro* culture. The significance values were indicated as ● between activated and activated+Dex cultures, and * between 24 and 72 h cultures. Dex was used in the following concentrations; 10^{-9} (10^{-9} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$, and similarly for ●).

Summary:

1. Dexamethasone did not affect the generation of nTregs in co-cultures of thymocytes and activated B cells.
2. The percentage of nTregs generated in co-cultures of thymocytes and activated B cells depended on the Thy : B ratio and B cell activation even in the presence of dexamethasone.

3.3.4. Evaluation of apoptosis of B cells in dexamethasone-conditioned medium

The percentage of apoptotic splenic B cells has been examined in 24 h cultures in the presence of dexamethasone to investigate the effect of different concentrations of Dex on the induction apoptosis in B cells and its potential to reduce the number of living cells in the cultures. The percentage of apoptotic cells strongly depended on the concentration of activators and Dex dose. Dex did not influence the level of apoptosis in B cells as compared to control (except from one concentration of Dex (10^{-12}M) that significantly decreased the percentage of apoptotic cells) (Fig. 3.32 A). The higher concentration of LPS and IMQ counteracted the effect of Dex significantly lowering the number of apoptotic B cells (Fig. 3.32 B and D). On the other hand, the weak signal delivered from low concentration of LPS cannot counteract the suppressive effect of Dex, furthermore, the concentration 10^{-8}M of Dex was shown to induce apoptosis (Fig. 3.32 C).

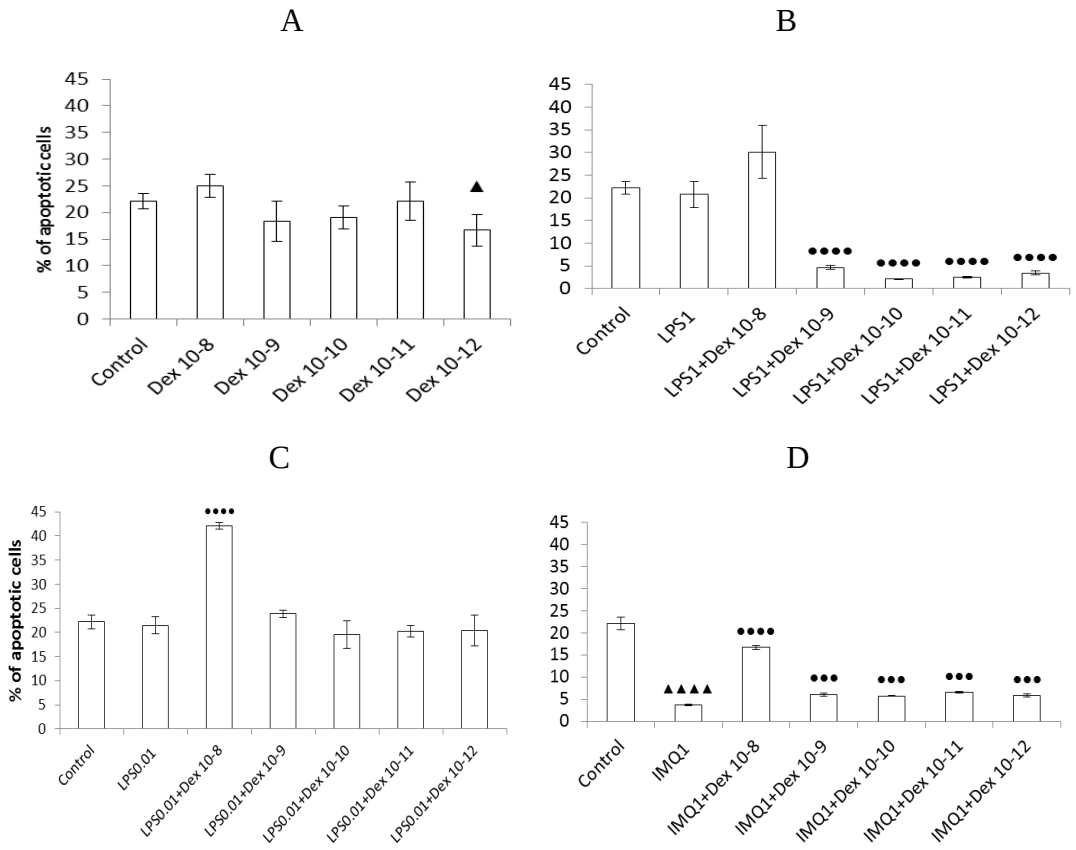


Figure 3.32:— Percentage of apoptotic splenic B cells in different conditions of culture.
The effect of Dex (A), LPS1+Dex (B), LPS0.01+Dex (C), and IMQ1+Dex (D) . The cells were analyzed after 24 h of *in vitro* culture.
The significance values were indicated as▲ between control and activated culture, and ● between activated and activated+Dex cultures. Control (non-activated cells), LPS1 (1µg/ml), LPS0.01 (0.01µg/ml), and IMQ1 (1µg/ml). Dex was used in the following concentrations; 10^{-8} (10^{-8} M), 10^{-9} (10^{-9} M), 10^{-10} (10^{-10} M), 10^{-11} (10^{-11} M), and 10^{-12} (10^{-12} M). The levels of probability were indicated as follows (▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; and ▲▲▲▲$p < 0.0001$, and similarly for ●).

The results show that Dex did not induce apoptosis in B cells with the exception of LPS0.01/Dex 10^{-8} M. Activation of B cells diminished the level of apoptosis. Thus, down-regulation of co-stimulatory molecules and MHC II did not result from the induction of apoptosis of B cells.

Summary:

1. Dexamethasone did not increase the level of apoptosis as compared to the control.
2. The activation of B cells by LPS and IMQ lowered the level of apoptosis in Dex- supplemented medium.
3. Low level of apoptosis after activation and Dex treatment depended on the concentration of LPS and was not observed for the lower concentration of LPS.

Discussion

Some immunosuppressive and anti-inflammatory agents have a potential to generate tolerogenic DCs in mice and humans, that in some conditions may lead to the induction of regulatory T cells (Adorini et al., 2004). Immature DCs have been found to have tolerogenic properties and can induce T cells with suppressive function (Jonuleit et al., 2000). It was documented that tolerogenic DCs should exhibit an immature phenotype characterized by decreased expression of co-stimulatory B7 (CD80 and CD86), MHC II, and functional activator (CD40) molecule, which can lead to an alternative antigen presentation and co-stimulation (Steinman et al., 2003; Ueno et al., 2007; van Kooten et al., 2009). In consequence tolerogenic DCs acquire the ability to establish tolerance through the promotion of regulatory T cell responses (Pulendran et al., 2010). Furthermore, these tolerogenic DCs can promote the differentiation and proliferation of T cells with regulatory functions (Matsue et al., 2002; Hackstein and Thomson, 2004; Unger et al., 2009; Huang et al., 2010; García-González et al., 2013). Different studies have shown that *in vitro* treatment of DCs with immunosuppressive agents leads to down-regulation of co-stimulatory molecules (CD80, CD86, and CD40), to decreased IL-12 synthesis, and enhanced IL-10 production, resulting in decreased T-cell activation (Berer et al., 2000; Griffin et al., 2000; Penna and Adorini, 2000, Piemonti et al., 2000). These properties highlight the important functional effects of immunosuppressive agents on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties (Adorini et al., 2004).

Dexamethasone is one of the most investigated immunosuppressive agents (Woltman et al., 2006; van Duivenvoorde et al., 2007). It is known as an immunosuppressive agent widely used to treat autoimmune diseases and prevent graft rejection (van Kooten et al., 2009; Moreau et al., 2012). The effects of Dex on the expression of different molecules and induction of apoptosis in different types of lymphocytes have been investigated. For instance, Dex causes a reduction of both splenic and lymph node B-cell numbers and attenuation of early B-cell progenitor proliferation, suppression of IgG and induction of IgE production (Baschant and Tuckermann, 2010; Dietrich et al., 2011). It was shown that glucocorticoids inhibit the production of IL-12, IFN-γ, IFN-α, and TNF-α by antigen-presenting cells (APCs) and T helper Th1 cells, but up-regulate the production of IL-4, IL-10, and IL-13 by Th2 cells

(Elenkov, 2004). Furthermore, it was found that Dex can inhibit the expression of CD40L on PMA/ionomycin-activated peripheral CD4+ T cells (Bischof and Melms, 1998), and up-regulate the expression of Foxp3 molecule *in vitro* (Prado et al., 2011). Consistent with these and other results, we aimed to use Dex as tolerizing agent to induce tolerogenicity in B cells in attempt to mirror the effect of Dex with DCs.

Our results revealed that Dex decreased the percentage of cells expressing all investigated molecules (CD80, CD86, CD40, and MHC II) (Fig. 3.22, 3.23, 3.24, and 3.25). These results were consistent with previously published data (Berer et al., 2000; Griffin et al., 2000; Penna and Adorini, 2000, Piemonti et al., 2000; García-González et al., 2013).

The results of our experiments showed that Dex did not change the distribution of main thymocyte subsets (Fig. 3.30).

The percentage of nTregs generated *in vitro* did not change in all conditions of co-culture in the presence of Dex (Fig. 3.31), showing that Dex did not increase the potential of the activated splenic B cell to generate thymus-derived Treg cells.

To check if the presence of Dex in co-cultures induces B-cell apoptosis and in consequence lower the number of the generated thymus-derived Treg cells, the apoptosis level in B cells after LPS-or IMQ-activation in splenocytes /24h has been studied. Our results revealed that the usage of different concentrations of Dex induced apoptosis in an acceptable percentage of B cells (less than 25%). Activation by LPS1 or IMQ1 significantly decreased the percentage of apoptosis induced by Dex (Fig. 3.32). These results indicated clearly that B cells remain viable in spite of administration of Dex, but Dex cannot induce tolerogenicity in B cells most probably due to the presence of other factors (LPS or IMQ). Due to the inhibition of Dex pro-apoptotic activity the percentage of generated nTregs in the co-cultures of thymocytes and B cells remained unchanges as shown in this section. This suggest that dexamethasone did not influence the tolerogenic potential of B cells, and the generation of nTregs depended on the presence of B cells, the level of their activation, and the thymocytes : B cells ratio.

4. Conclusions

Our hypothesis on the role of B cells in the generation of thymus-derived Treg cells was built on the following facts: firstly, the normal development of nTreg in the thymus requires interaction with thymic antigen-presenting cells such as dendritic cells and thymic epithelial cells. These cells are crucial for the selection and maturation of thymus-derived Treg cells. Secondly, it was found that a minor percentage of B cells reside in the thymus, and the origin and the role of these cells are still poorly studied. Thus, to investigate if B cells have the potential to induce the generation of thymus-derived Treg cells *in vitro*, we introduced a model of co-culture of thymocytes with splenic B cells. Splenic B cells have been used based on the observation of the migration of peripheral B cells into the thymus, and thymocytes have been used as the source of precursors of nTregs. Based on the results of the study presented in this dissertation we can conclude:

1. Splenic B cells can induce the generation of thymus-derived Treg cells *in vitro* and the percentage of newly generated nTregs is increased upon activation by LPS or IMQ.
2. The percentage of thymus-derived Treg cells generated *in vitro* was dependent on the thymocytes : B cells ratio.
3. nTreg cells generated *in vitro* in co-culture with B cells exhibited the suppressive activity.
4. MHC II and CD80/CD86 molecules expressed on splenic B cells play a major role in thymus-derived Tregs generation compared to CD40 molecule.
5. Thymic B cells activated by LPS or IMQ sustained the viability of thymus-derived Treg cells *in vitro* by inducing the proliferation of thymic B cells upon activation thereby increasing their percentage in the culture and thymocytes : B cells ratio.
6. Dexamethasone did not affect the generation of thymus-derived Treg cells in the co-culture with splenic B cells.

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